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The genetic analysis of bHLH-PAS genes in *Caenorhabditis elegans*

by

Huaqi Jiang

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ABSTRACT

The family of basic-helix-loop-helix-PAS (bHLH-PAS) transcription factors mediate diverse cellular processes. These include several important cell fate decisions and cellular responses to environmental signals. Molecular and biochemical data indicate that *C. elegans ahr-1* and *aha-1* encode the orthologs of the mammalian aryl hydrocarbon receptor (AHR) and the AHR nuclear translocator (ARNT). The *C. elegans* genome also encodes 3 other bHLH-PAS proteins, *hif-1*, *cky-1* and T01D3.2. In this dissertation, I describe molecular, biochemical, and genetic analyses of *hif-1* and *aha-1* and their gene products. *C. elegans hif-1* encodes the homolog of the mammalian hypoxia-inducible factor α subunit. They form a complex that is similar to the mammalian hypoxia-inducible factor, and *hif-1* mediates hypoxic responses in *C. elegans*. *hif-1* mutants are viable at standard laboratory conditions, but they are unable to adapt to hypoxia. Wild-type animals can survive and reproduce in 1% oxygen, but the majority of the *hif-1*-defective animals die in these conditions. We show that HIF-1:GFP expression is induced by hypoxia and is rapidly degraded upon reoxygenation. HIF-1 co-immunoprecipitates with AHA-1 *in vitro*. We conclude that the hypoxia-inducible factor complex is conserved in *C. elegans*. To better understand the role of *aha-1* in *ahr-1* and *hif-1* function, I initiated a genetic analysis of *aha-1* function. *aha-1* deletion mutants arrest as larvae and are defective in feeding. The larval arrest phenotype of *aha-1* mutants is much more severe than the *ahr-1*, *hif-1* double mutants (which are viable), suggesting that *aha-1* has additional functions. We analyzed the expression patterns of the two remaining bHLH-PAS genes, T01D3.2 and *cky-1*. A T01D3.2:GFP reporter is expressed in two interneurons. *cky-1* may represent a new bHLH-PAS gene subfamily, and a CKY-1:GFP reporter is expressed mainly in the pharynx, the feeding organ of the worm. These data suggested that AHA-1 and CKY-1 might interact and function in the pharynx. Several lines of evidence support this model. AHA-1 and CKY-1 form a DNA-binding complex *in vitro*. AHA-1 is nuclear localized in the subset of pharyngeal cells that express CKY-1:GFP. Most convincingly, expression of *aha-1* from the *cky-1* promoter is sufficient to rescue the *aha-1* larval arrest phenotype. We propose that *aha-1* has an essential function in the *C. elegans* pharynx.

CHAPTER 1 GENERAL INTRODUCTION

The ability to recognize and respond to developmental and environmental signals is essential for all life forms. In many cases, cells respond to extracellular signals by changing the activity of particular transcription factors. The resultant changes in gene expression control many important cellular responses, such as cell differentiation, proliferation, apoptosis and migration. The class of transcription factors containing basic, helix-loop-helix, and PAS motifs (bHLH-PAS proteins) regulate an impressive range of cell fate decisions. In this dissertation, I study the function of bHLH-PAS proteins in a powerful genetic system, the nematode *Caenorhabditis elegans*. I demonstrate that bHLH-PAS proteins regulate cellular responses to hypoxia and have other essential functions during *C. elegans* development.

LITERATURE REVIEW

The model organism Caenorhabditis elegans

The soil nematode *Caenorhabditis elegans* was originally selected by Sydney Brenner in 1963 as a simple genetic model organism to study metazoan development, behavior and the function of the nervous system (1). Its short life cycle (3 days at 25°C) and the two sexes, hermaphrodite and male, make it an ideal organism for developmental genetics. In the laboratory, *C. elegans* can be cultured conveniently on agar plates with a bacterial food source. Mutants can be easily obtained through chemical mutagenesis or ionizing radiation. Mutant strains can be frozen and stored.

The *C. elegans* life cycle and anatomy are well studied. The hermaphrodite can reproduce by self-fertilization or by mating with males. Fertilized eggs undergo embryogenesis and hatch as first stage larvae. After a series of four molts and three additional larval stages, they become fertile adults. A fully-grown adult is about 1mm long. At this stage, hermaphrodites contain 959 somatic nuclei, and males have 1,013 somatic nuclei. The complete cell lineage has been determined, and it is essentially invariant (2, 3). The *C. elegans* anatomy has been determined by reconstruction of electron micrographs of serial sections. This data is complemented by a wealth of subsequent expression studies, and

it provides detailed information about every cell's differentiated state. Extensive descriptions of the positions of neuronal processes and cell bodies have enabled the construction of neuronal connectivity diagrams (4-6).

A recent milestone for *C. elegans* research was the release of the essentially complete genomic sequence by the *C. elegans* Sequencing Consortium in 1998 (>99% complete today)(7). The worm genome is predicted to contain about 19,000 genes, and over 17,300 of them have been confirmed as transcribed genes (8). 43% of worm genes have apparent human homologs (9). The *C. elegans* sequence is well annotated (10), and it has provided the foundation for the development of *C. elegans* functional genomics.

The post-genomic era of *C. elegans* genetics has been characterized by the increased use of reverse genetics, a method in which researchers generate mutations in known or predicted gene sequences. The *C. elegans* Knockout Consortium has generated deletions in hundreds of genes using chemical mutagenesis. The deletions were identified and subsequently isolated by PCR-based protocols (http://elegans.bcgsc.bc.ca/knockout.shtml, 11).

Genome wide studies of gene function have been initiated using RNA-mediated gene interference (RNAi). This technique is based on a recent discovery that a gene-specific double stranded RNA (dsRNA) can cause the degradation of the corresponding mRNA, resulting in loss-of-function phenotypes (12, 13). dsRNA can be delivered into the worm by several methods, such as injecting dsRNA into the syncytial gonad of the hermaphrodite (13), soaking the worm in the dsRNA solution (14), or feeding the worm bacteria that express dsRNA (15). High throughput RNAi studies of worm genes have been reported (16-19), and the results are entered into Wormbase, the online *C. elegans* genomic database. Initial analysis of the genes on chromosome I indicated that RNAi caused a visible phenotypic defect for 13.9% of the genes assayed. Included in this analysis were some genes that had been defined by mutational studies. In 31 of 62 cases, the loss-of-function phenotype was also revealed by RNAi (16). A modified dsRNA soaking method was used to assay 2,479 randomly selected genes, and phenotypic abnormalities were observed for 27% of the genes targeted (19). Those numbers probably underrepresented the loss-of-function phenotypes for the targeted genes, because RNAi is transient. RNAi has also been shown to

be relatively ineffective in neurons (20, 21). In general, genes with essential embryonic functions are much more likely to be identified by RNAi than those with a postembryonic function (22).

The essentially invariant *C. elegans* cell lineage can, in theory, be combined with genome-wide expression studies to compile and compare cellular transcriptomes. Two different methods, gene reporters (GFP or *LacZ*) and whole-mount *in situ* hybridization, are currently used to analyze gene expression patterns in large-scale studies (http://nematode.lab.nig.ac.jp). This information is further refined and expanded by directed and thorough analyses of gene expression in individual laboratories.

DNA microarrays are used to examine the expression profiles of *C. elegans* genes at specific developmental stages, different growth conditions, or varying genetic backgrounds. Affymetrix chips containing oligonucleotides specific for most of the predicted *C. elegans* open reading frames (ORFs) were used to compare gene expression profiles in 8 stages of development, from oocytes to 2-week old adults (24). Expression was detected for 56% (10,741/18,791) of ORFs analyzed. Using DNA chips containing genomic PCR products corresponding to 11,917 *C. elegans* genes, Reinke et al. (2000) detected expression of 59% of the genes assayed. They compared gene expression levels between wild-type and germline-defective mutants and identified 1,416 genes that are enriched in the germline. Further, they were able to identify oocyte and/or sperm-enriched genes (25). Using similar techniques, Jiang et al. (2001) identified 2,171 male- or hermaphrodite-enriched genes (26). Additionally, they identified 25 groups of genes whose expression covaried during development. Analysis of a much larger data set, microarray data from 553 experiments, allowed the identification of 43 groups of genes whose expression covaried under a variety of conditions (27). Genes in the same group are regulated in a similar way, suggesting that they may be functionally related. One of the limitations of *C. elegans* microarray experiments is that only about 60% of the genes are detected above background level, presumably because some genes are expressed at levels that are too low to be measured by the DNA microarrays.

Another functional genomics project is to generate a protein-protein interaction map of all *C. elegans* predicted ORFs using a genome-wide yeast two-hybrid library. Twenty-seven genes known to regulate vulval development were used in pilot experiments, and 150

interactions were detected (28). This high-throughput method detects approximately half of the protein-protein interactions that have been discovered in other studies. Still, it will identify a lot of biological interactions, especially when combined with other genome-wide studies, such as large scale of phenotypic analysis by RNAi (29).

All these new functional genomics approaches have already had a great impact on how individual researchers design experiments. Both forward and reverse genetic approaches are available to study certain developmental processes or the function of a particular gene. Traditional positional cloning of a gene identified by mutation is made much easier by both the detailed physical map and the genomic sequence. A growing high density single-nucleotide polymorphism (SNPs) map of *C. elegans* also aids positional cloning (30, 31). When studying the function of a particular gene, testable hypotheses may come from functional genomics data, including genetic interactions, coregulation with other genes, physical interactions of the gene product, and also the cells in which it expressed.

basic helix-loop-helix-PAS (bHLH-PAS) proteins

The changes in gene expression that direct development are controlled by transcription factors. The family of transcription factors that contain basic, helix-loop-helix, and PAS motifs (bHLH-PAS proteins) regulate an impressive range of cellular differentiation events and adaptations to environmental cues (32, 33).

The PAS domain is a conserved protein motif originally identified in *Drosophila Period* (*Per*), mammalian aryl hydrocarbon receptor nuclear translocator (ARNT) and *Drosophila Single-minded* (*Sim*) (34). *Per* is involved in *Drosophila* circadian rhythm regulation (35). ARNT was first isolated as the co-factor required for aryl hydrocarbon receptor (AHR) signaling (36). *Sim* mediates the development of the *Drosophila* central nervous system (CNS) (34). The PAS domain consists of 250-300 amino acids which contains two imperfect repeats, called PAS core domains, A and B.

Recently the predicted structure of a region containing ARNT PAS B core domain was shown to be very similar to the crystallographic structure of the bacteria photoactive yellow protein (PYP) (37), the heme domain of the FixL protein (38), and the N-terminal domain of the eukaryotic potassium channel HERG (39). In eukaryotes, the PAS domain

mediates protein-protein interactions (see below for more detail) and ligand binding, such as ligands for the mammalian aryl hydrocarbon receptor (AHR). Eukaryotic PAS domain proteins can either directly sense environmental changes (AHR directly binds to its ligands) or transduce environmental signals from upstream sensors (hypoxia-inducible factor-1α in hypoxic responses or Per in circadian rhythm regulation by light). Prokaryotic PAS domain is required for cofactor binding in some proteins, such as cofactors for PYP and FixL. PYP is a blue-light photosensor, and the blue light is captured by the 4-hydroxycinnamyl chromophore within the PAS domain (40). FixL is an oxygen sensor, oxygen directly binds to heme within the PAS domain (41). Thus the PAS domain is a multi-functional interaction domain conserved from bacteria to human, and the PAS superfamily is proposed to function as sensors of environmental or developmental signals (32, 33, 42).

With the exception of PER and its homologs, PAS domain-containing proteins that have been identified in eukaryotes also contain a basic helix-loop-helix (bHLH) domain at the N-terminus. Hence, they are called bHLH-PAS proteins. They are transcription factors that function as heterodimers, and heterodimer formation is required for their activities, including DNA binding and transactivation. The structure of bHLH-PAS proteins, exemplified by ARNT and its homologs, *Drosophila* Tango and *C. elegans* AHA-1, is shown in Figure 1. The N-terminal basic domain is the DNA binding domain. Each basic domain in a dimer recognizes its own half site. Thus the DNA binding specificity of the dimer is determined. The helix-loop-helix domain is required for the interaction between two bHLH-PAS proteins, and it forms the interface between two bHLH-PAS proteins (43). However efficient heterodimer formation also requires the PAS domain. Deletion of the ARNT PAS domain significantly reduces its ability to dimerize with AHR (43). Furthermore, the PAS domains of ARNT and AHR interact directly, even in the absence of the helix-loop-helix domain (44). Thus, multiple domains of ARNT are required for the maximal heterodimerization with AHR. A truncated AHR containing only the bHLH domain can form dimers with multiple proteins containing HLH motifs, but the full-length AHR can not, demonstrating that the PAS domain also confers dimerization specificity (45). Besides its role in heterodimer formation, the PAS domain of *Drosophila* Trachealess (Trh) also confers target gene specificity. When the *Drosophila* Single-minded (Sim) PAS domain is exchanged

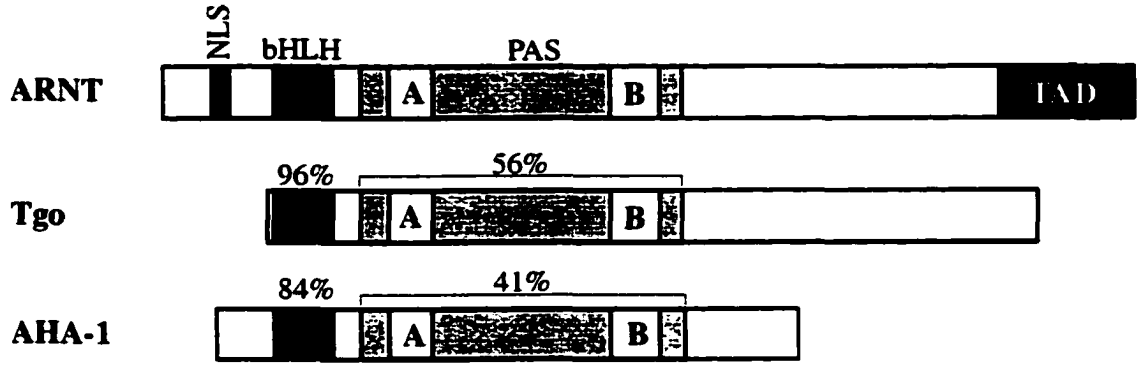

Figure 1 Structure of ARNT bHLH-PAS protein subfamily. Shown are ARNT, mammalian aryl hydrocarbon receptor nuclear translocator (ARNT); Tgo, *Drosophila* Tango protein; AHA-1, *C. elegans* AHA-1 protein. The nuclear localization signal (NLS) is only found in the mammalian ARNT. The conserved basic-helix-loop-helix (bHLH) domains are represented by black boxes. The Per-Arnt-Sim (PAS) domains are indicated by gray boxes, which contain two imperfect repeats called PAS core domains, A and B. The percentage of identities of Tgo and AHA-1 in the bHLH and PAS domain, as compared with ARNT, are indicated. The transcriptional activation domain (TAD) has been functionally defined in ARNT, but not in Tgo or AHA-1. Their protein sequences C-terminal of the PAS domain are not conserved.

for the Trachealess (Trh) PAS domain, the chimeric protein activates Trh target genes (46). AHR deletion studies also revealed that the PAS domain is required for interaction with chaperones (Hsp90) or ligands, such as polycyclic aromatic hydrocarbons (PAHs) and dioxin (47). As transcription factors, most bHLH-PAS proteins contain transcriptional activation domains (TAD) at the C-terminus. However, their C-terminal sequences have little similarities. Finally, certain domains are specific for individual bHLH-PAS proteins, such as the oxygen-dependent degradation domain (ODD) of the hypoxia-inducible factor α (HIFα) (48).

ARNT proteins are common dimerization partners for multiple bHLH-PAS proteins

Every DNA-binding dimeric complex of bHLH-PAS proteins that has been described thus far contains ARNT or one of its close homologs. Thus, the ARNT subfamily has many developmental roles, and the other bHLH-PAS proteins are dedicated to specific regulatory functions. Both mammalian ARNT and its *Drosophila* homolog Tango (Tgo) are able to dimerize with multiple bHLH-PAS proteins (Figure 2).

One of the most extensively studied bHLH-PAS complexes consists of mammalian ARNT and AHR. In the absence of ligand, mammalian AHR is primarily cytosolic in a complex with heat-shock protein Hsp90 and another chaperone ARA9 (also known as AIP1 or XAPs). Upon binding to its ligand, AHR translocates into the nucleus where it dissociates from the chaperones and binds ARNT. The ARNT/AHR heterodimer binds specific dioxin responsive elements (DRE) on the promoter of target genes to regulate transcription. The target genes include a battery of xenobiotic metabolizing enzymes (XMEs), including cytochrome P450-dependent monooxygenases, such as CYP1A1 and CYP1A2. These enzymes are involved in the metabolism of polycyclic aromatic hydrocarbon (PAHs), including benezo[a]pyrene. The resulting intermediate metabolites damage DNA, and are, therefore, carcinogenic (49). AHR knockout mice are resistant to the carcinogenecity of benzo[a]pyrene, demonstrating that AHR is involved in mediating the carcinogenic activity of PAHs (50). AHR also has been shown to play important roles in mouse development. Ahr null mice generated in three independent laboratories exhibit defects in liver development, low weight and poor fertility (51-53). Recently Ahr-/- mice were shown to have defects in

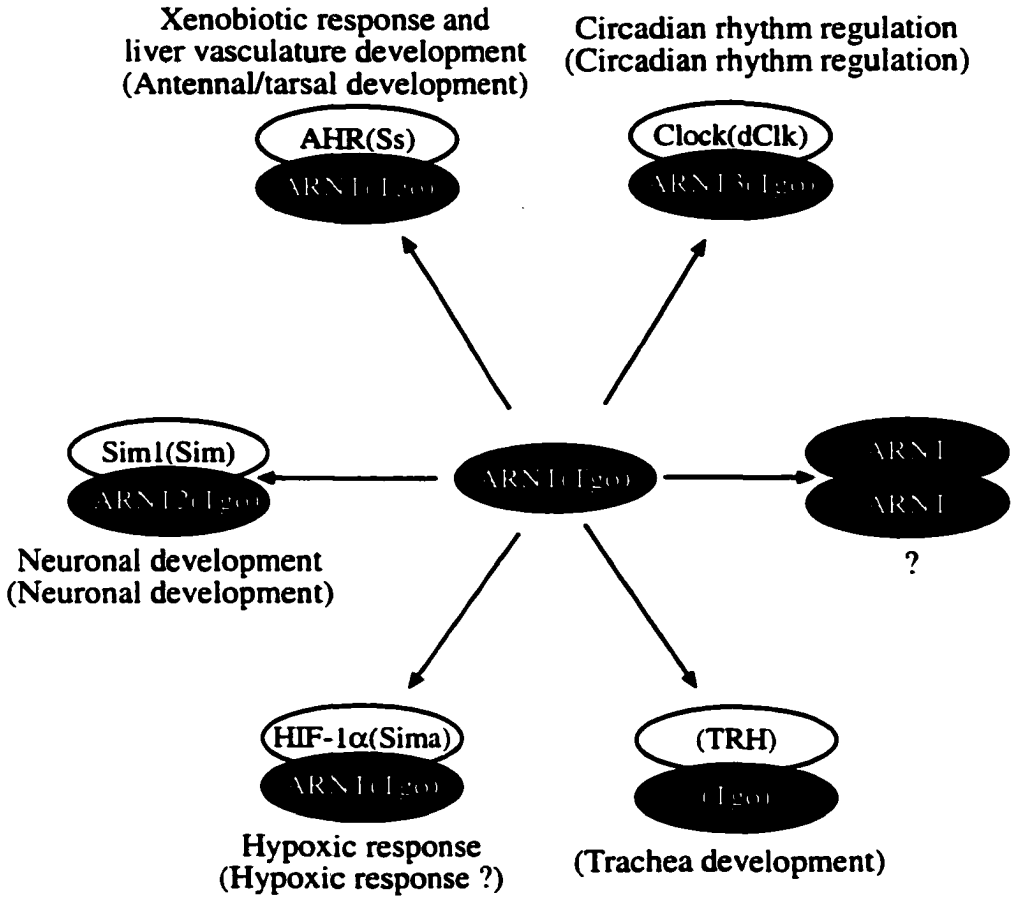

Figure 2 ARNT is the common dimerization partner for multiple bHLH-PAS domain proteins. ARNT and its homologs (represented by the black ovals) form dimers with partners dedicated to specific cellular or developmental functions. The examples depicted here are the aryl hydrocarbon receptor (AHR), circadian rhythm protein Clock, hypoxia-inducible factor-1α subunit (HIF-1α) and mammalian single-minded homolog Sim1. ARNT also forms a homodimer. *Drosophila* Tango (Tgo) is the homolog of ARNT. Tgo is able to interact with the AHR homolog Spineless-aristapedia (Ss), Single-minded (Sim), Trachealess (Trh) and *Drosophila* HIF-1α homolog Similar (Sima). Cycle (Cyc) is the *Drosophila* Arnt3 homolog. Cyc interacts with the *Drosophila* Clock homolog (dClk). The functions of the dimers are indicated. *Drosophila* homologs and their functions are listed in the parentheses. ? indicate that the function either is unknown (ARNT homodimer) or has been suggested, yet still has not been proven (*Drosophila* Sima/Tgo dimer).

ovary development (54, 55). Interestingly, PAHs exposure also causes defects in mouse ovary development. The toxicity is dependent on the function AHR and its target gene Bax (56). AHR is evolutionary conserved (57-59). The *Drosophila* AHR homolog is encoded by the *spineless-aristapedia* (*ss*) gene and it interacts with Tgo. *Ss* plays important roles during development. *ss* mutants have smaller sensory cells (bristles), and the distal leg (tarsus) is deleted. Additionally, the distal antenna is transformed into distal leg (59). It has been suggested that chemosensation was the ancestral role of AHR/Ss (60).

The *Drosophila* bHLH-PAS protein Sim also interacts with Tgo, and the heterodimer controls the central nervous system (CNS) midline development (34) (Figure 2). Mammalian Sim1 and Sim2 are able to form similar complexes with ARNT and its close homolog ARNT2 (61). Mutant mice lacking Sim1 and ARNT2 die shortly after birth (62-65). The similar defects in the differentiation of secretory neurons (such as paraventricular nucleus, PVN) in the hypothalamus suggest that ARNT2 is the likely *in vivo* dimerization partner for Sim1 during the development of neuro-endocrinological cell lineages of the hypothalamus and pituitary gland. Interestingly, mouse Sim1 haploinsufficiency also causes obesity, apparently resulting from the decreased number of paraventricular nuclei (PVN) in the hypothalamus of affected mice (66). A human balanced translocation that disrupted the Sim1 gene was described recently in a patient with severe early-onset obesity (67).

ARNT3 (also known as BMAL1, MOP3) is a close homolog of mammalian ARNT, and it interacts with another bHLH-PAS protein CLOCK (Figure 2) (68-70). They form the positive regulator of the molecular feedback loop in the circadian pacemaker (or clock) (71-75). Clock and MOP3 loss-of-function mice are arrhythmic (76-78). *Drosophila* Clock and Cycle (MOP3 homolog) form a similar complex, and *clock* and *cycle* mutants are also arrhythmic (73, 74).

Drosophila Trachealess (*trh*) controls the formation of certain tubular structures. In *trh* mutants, epithelial tube-forming cells of the salivary gland, trachea, and filzkorper fail to invaginate and remain on the surface of the embryo (79). Trh functions as a heterodimer with Tgo. Trh is also regulated by *Drosophila* Akt. Phosphorylation of S665 by dAkt is required for Trh nuclear localization (80).

Drosophila tgo mutants are embryonic lethal, and they have defects similar to those in *sim* and *trh* mutants (81). *tgo* mutants also have been isolated as enhancers of *ss* mutations (60). These data support the conclusion that Tgo is the common dimerization partner for Ss, Sim and Trh in *Drosophila* (Figure 2).

Mammalian ARNT can form homodimers *in vitro*, and the homodimer is able to bind to a class B E-box enhancer containing two adjacent ARNT binding half-sites (5'-CACGTG) (82-84). Additionally, studies in mammalian cell lines have demonstrated that this enhancer can direct expression of a reporter gene in an ARNT-dependent manner (82, 83). However the *in vivo* targets and functions of ARNT homodimers are not clear.

Although mammalian ARNT and its known invertebrate homologs share high sequence similarities, their regulation is different. Mammalian ARNT contains a nuclear localization sequence (NLS) at its N-terminus (85), and this motif is absent in *Drosophila* Tgo and *C. elegans* AHA-1 (Figure 1). Current models suggest that ARNT is constitutively nuclear localized (85). However efficient nuclear localization of Tgo requires the presence of their dimerization partners (86). In *C. elegans*, efficient nuclear localization of AHA-1 in the intestinal cells is dependent on the *hif-1* function (87).

Mammalian ARNT is phosphorylated at multiple sites (88). Dephosphorylation of ARNT reduces its ability to form heterodimers with AHR *in vitro* (89). Protein Kinase C (PKC)-dependent phosphorylation events positively regulate AHR signaling activity in some cell types (89-91). Furthermore, PKC also has been shown to modulate ARNT homodimer transactivation activity, as assayed by a reporter containing the class B E-box enhancer (92).

Hypoxia response pathway

Molecular cloning of hypoxia-inducible factor-1

Oxygen homeostasis is critical for the survival of most aerobic life forms because of its role in mitochondrial oxidative phosphorylation, which generates ATP. When mammals are exposed to hypoxia, cells or tissues must adapt by increasing oxygen supply and reducing reliance on oxygen for energy metabolism. Ischemic tissue damage resulting from some of the most common human diseases, such as heart attack, stroke and pulmonary hypertension, are the leading causes of mortality. Hypoxia also plays important roles in cancer

development. Cells in a tumor mass must overcome hypoxia-induced apoptosis (93). They also respond to hypoxia by secreting growth factors that induce angiogenesis (94).

The role of bHLH-PAS proteins in regulating hypoxia response was revealed by the molecular identification of hypoxia-inducible factor-1 (HIF-1) (95). Erythropoietin (EPO) is a well-characterized hypoxia-inducible gene (96, 97). Oxygen-dependent induction of EPO transcription requires a hypoxia-responsive element (HRE, 5'-RCGTG) in its promoter (98, 99). Purification of the factor bound to EPO HRE revealed that HIF-1 contains two subunits, HIF-1α and HIF-1β (100). The protein sequence of HIF-1β was shown to be identical with that of ARNT (95). HIF-1α encodes a bHLH-PAS protein that is dedicated to hypoxia response (95). Other alpha subunits (mammalian HIF-2α (EPAS1) and HIF-3α) and β subunits (ARNT2 and BMAL1/MOP3/ARNT3) have also been isolated and they are expressed in certain tissues (70, 101-103).

Model of hypoxia signaling

HIF-1 activity is regulated by oxygen concentration at multiple levels. During normoxia, HIF-1α and HIF-1β are both transcribed, and HIF-1α mRNA is up-regulated over 10-fold in rat brain and lung tissue after hypoxia treatment (104, 105). However in cell culture experiments, the stability of HIF-1α protein is the most important regulatory mechanism. The model for HIF-1α regulation is illustrated in Figure 3. Translated HIF-1α is rapidly degraded by an ubiquitin-proteasomal pathway at normoxia (ambient oxygen concentration, 21%) (48, 106, 107). Under hypoxic conditions, HIF-1α ubiquitination is reduced and its degradation is inhibited (106, 108). When oxygen levels are high, a conserved proline residue (Pro564) within the oxygen-dependent degradation domain (ODD) of HIF-1α is hydroxylated (109, 110). The modified HIF-1α is recognized by the Von Hippel-Lindau tumor suppressor protein (VHL), which is a component of ubiquitin ligase complex. VHL binds to the hydroxylated HIF-1α and targets it for ubiquitination. Polyubiquitinated HIF-1α is subsequently degraded by the 26S proteasome (111). The enzyme and putative oxygen sensor that modifies the ODD was first identified in *C. elegans*, and it is encoded by the *egl-9* gene (112). The mammalian homologs of *C. elegans* EGL-9 encode the prolyl-4-hydroxylases that modify HIF-1α (112, 113). They are Fe(II)- and 2-

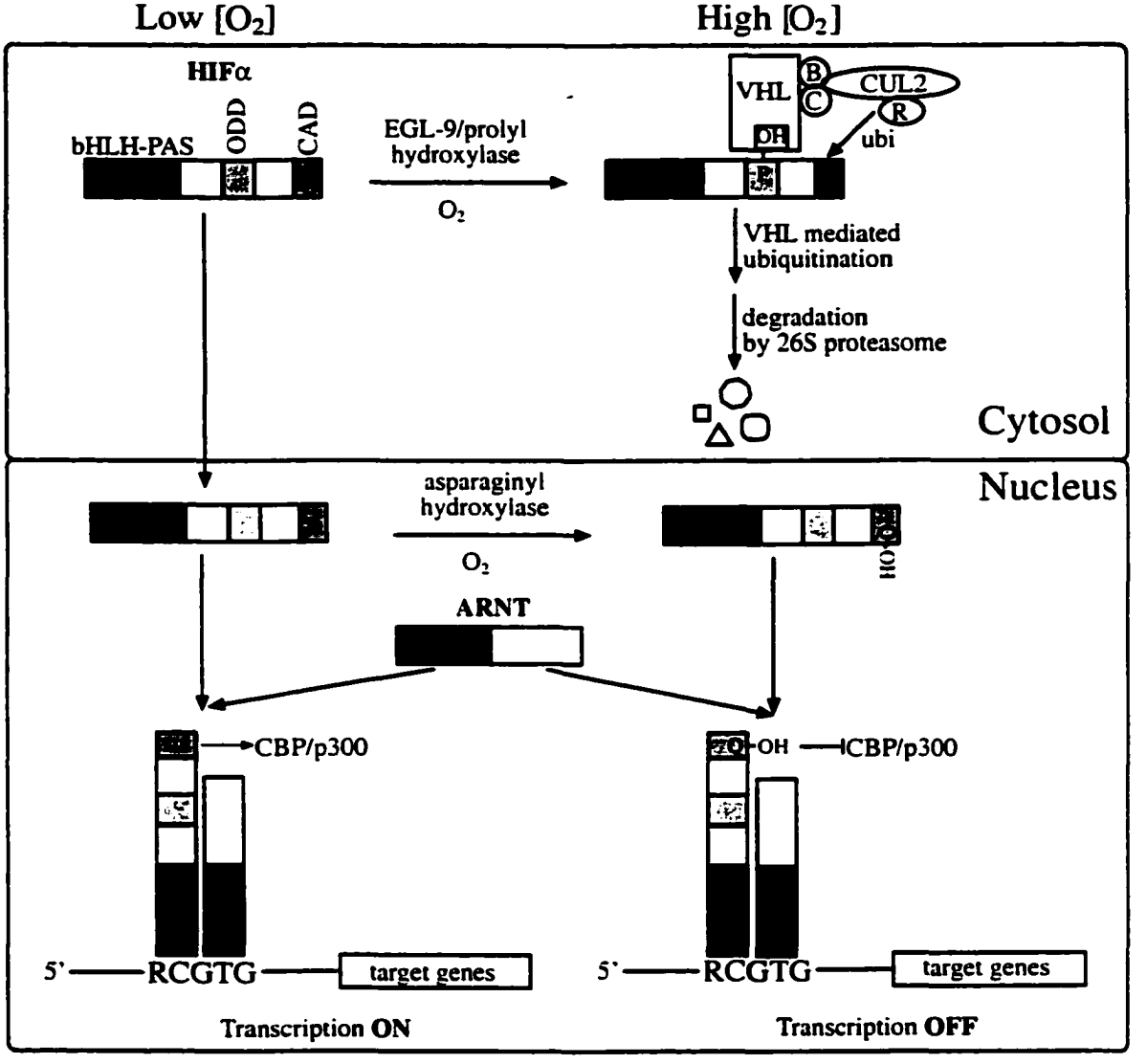

Figure 3 Model for the regulation of hypoxia-inducible factor (HIF). Under normoxic conditions. The HIFα oxygen-dependent degradation domain (ODD) is hydroxylated by prolyl hydroxylases. Modified HIFα is recognized by the Von Hippel-Lindau (VHL) tumor suppressor protein, which recruits elongins B and C (B, C), Cullin-2 (CUL-2) and RBX1 (R) to ubiquitinate HIFα. Polyubiquitinated HIFα is subsequently degraded by the 26S proteasome. Hypoxia inhibits the EGL-9/prolyl hydroxylase and blocks the degradation of HIFα. Thus, HIFα protein accumulates and translocates to the nucleus where it dimerizes with ARNT to form the HIF complex. HIF binds to the hypoxic response element (HRE, 5' RCGTG) on the promoter of the target genes. HIFα COOH-terminal transactivation domain (CAD) recruits transcriptional coactivators such as CBP/p300 and activates the transcription of the target genes. The recruitment of CBP/p300 is also regulated by oxygen levels. At normoxia, CAD is hydroxylated by an asparaginyl hydroxylase, which blocks its interaction with CBP/p300. Thus, both the stability and the transcriptional activity of HIFα are regulated by oxygen levels.

oxoglutarate-dependent dioxygenases. Since oxygen is one of the substrates for these enzymes, Pro564 is hydroxylated only when oxygen is available. When oxygen is low, HIF-1α is not hydroxylated, and VHL does not target it for degradation. Thus HIF-1α accumulates and translocates to the nucleus, where it binds to its dimerization partner ARNT to form the active HIF-1 complex. HIF-1 binds to hypoxia response elements (HREs) on the target gene promoters, and recruits transcriptional cofactors such as CBP/p300 to regulate target gene transcription (114).

The recruitment of CBP/p300 by HIF-1 is also regulated by cellular oxygen levels. This is achieved, at least in part, by modifying HIF-1α at a second position, an asparagine residue (Asn803) within the COOH-terminal transactivation domain (CAD) (115). Asn803 is hydroxylated in an oxygen-dependent manner by an unidentified Fe(II)- and 2-oxoglutarate-dependent dioxygenase. Hydroxylation at Asn803 prevents CBP/p300 recruitment by the HIF-1α CAD. The two different hydroxylases are speculated to activate HIF-1 at different levels in response to the changing oxygen concentration in the environment (116).

Activated HIF-1 induces the expression of target genes to restore oxygen homeostasis. Those genes function at two different levels in mammals. On the systemic level, HIF-1 induces the expression of erythropoietin (EPO) and vascular epithelial growth factor (VEGF). EPO is a haematopoietic cytokine that increases red blood cell count, thus generating more oxygen carriers to increase oxygen supply. VEGF induces the formation of new blood vessels to increase the blood flow into the hypoxic tissue. On the cellular level, HIF-1 increases the expression of several glycolytic enzyme genes and glucose transporter genes, which function to increase glucose uptake and glycolysis. Enhanced cellular glycolysis reduces a cell's dependence on oxygen for energy metabolism (mitochondrial oxidative phosphorylation requires oxygen to generate ATP. Glycolysis does not). A more comprehensive review of HIF-1 target genes and physiological responses to hypoxia can be found elsewhere (117, 118).

Other regulators of HIF-1

HIF-1α degradation is also regulated by p53. p53 binds to HIF-1α and recruits the ubiquitin-protein ligase MDM2, which targets HIF-1α for ubiquitination and degradation

(119). Loss of p53 in certain cancer cells enhances the induction of HIF-1α and VEGF by hypoxia. On the other hand, p53 expression is induced by hypoxia, and the induction is HIF-1α-dependent (120). HIF-1α-mediated stabilization of p53 may play a role in hypoxia-induced apoptosis (93, 120).

Other signal transduction pathways also regulate HIF-1 activity. Like hypoxia, many growth factors, including insulin, IGF, EGF, PDGF, heregulin and IL-1β, have been reported to induce HIF-1 activity in a variety of cell lines (121-125). In many cases, the induction is dependent upon phosphatidyl inositol 3-kinase signaling (PI3K). In some cell lines, elevated PI3K pathway activity can facilitate the increase of HIF-1α expression at hypoxia. Reduced activity of the pathway blocks HIF-1α expression (126). Regulation of HIF-1 by growth factors appears to be cell type-specific (127).

Other factors regulate HIF-1 activity by directly binding to HIF-1α and affecting the heterodimer's DNA binding or transcriptional activity (128, 129). FIH-1 was identified in a yeast two-hybrid screen using HIF-1α CAD as a bait. FIH-1 binds to HIF-1α and VHL. VHL recruits histone deacetylases to repress HIF-1 transcriptional activity (128). Inhibitory PAS (IPAS) is another protein that directly interacts with HIF-1α. IPAS is a bHLH-PAS protein that lacks the C-terminal transactivation domain. It functions as a dominant negative regulator of HIF-1 by binding to HIF-1α and interfering with HIF-1 DNA binding. IPAS is mainly expressed in the cornea, and it functions to maintain an avascular structure there (129).

Genetic analysis of ARNT and HIFα

The significance of HIF-1 function is underscored by its essential role during mouse development, which is revealed in ARNT and HIF-1α knockout experiments. ARNT-deficient mice die before E10.5 and show defective angiogenesis in the yolk sac, branchial arches and embryonic placenta (130, 131). HIF-1α knockout mice die at E10.5 and have defective vasculature in the myocardial and cephalic tissues (132, 133). The vasculature defects of ARNT and HIF-1α mutants are very similar to those seen in VEGF mutants, providing further evidence that HIF-1 regulates VEGF expression *in vivo*. However, the role of VEGF in causing vascular defects in HIF-1α null mice has been questioned. Kotch et al.

(134) demonstrate that the VEGF level in HIF-1α null mice is increased. They suggest that mesenchymal cell death prior to vascular regression in HIF-1α null mice may be the cause for the defective vascular structure.

HIF-2α is a close homolog of HIF-1α. Both proteins are regulated by hypoxia. However, their expression patterns are different. HIF-2α is expressed predominantly in epithelial cells (101, 135). More than half of HIF-2α-deficient mice die between E9.5 and E13.5 with severe yolk sac vascularization defects (136). Another independently generated HIF-2α knockout mice strain dies at midgestation without vascular defects. Thus, the role of HIF-2α during vascular development is uncertain. Phenotypic analysis revealed that HIF-2α is essential for catecholamine homeostasis (137). Loss of HIF-2α protects ES cells against apoptosis during hypoglyceria, but not hypoxia. Further, HIF-2α$^{-/-}$ ES cells fail to induce VEGF and other angiogenic factors in response to hypoxia, but they do increase expression of glycolytic enzymes (138).

It has been reported that tumor cells, especially those of solid tumors, are generally hypoxic (139). Hypoxia induces vascularization of the tumor, which provides oxygen and other nutrients for tumor growth. Tumors derived from ES cells lacking HIF-1α do not vascularize well (94, 133). ARNT-deficient hepatomas have similar defects (140). Surprisingly, in one study, the growth of HIF-1α$^{-/-}$ ES tumor xenograph is not retarded, but increased. This is explained in part by the failure of tumor cells to undergo hypoxia-mediated cell death (94).

ARNT2 is expressed at high levels in the brain (135). ARNT2 knockout mice die shortly after birth because of defective hypothalamus development (62, 63). Induction of target genes by hypoxia is decreased in cultured ARNT2$^{-/-}$ neurons, suggesting it has partial redundancy with ARNT (62).

Oxygen sensors

The identification of EGL-9 and the homologous mammalian PHDs (prolyl hydroxylase domain-containing genes) provides the first direct link between oxygen concentration and HIF activity. The prolyl hydroxylases encoded by these genes require oxygen, iron and 2-oxoglutarate as cofactors or substrates. Oxygen is the rate limiting factor

for the activities of PHDs (112). This oxygen-sensing mechanism is consistent with the finding that HIF-1α accumulation is immediate upon exposure to hypoxia (141). Although the asparaginyl hydroxylases that modify the CAD have not been identified, biochemical analyses indicate that they belong to the same family of Fe(II)- and 2-oxoglutarate-dependent dioxygenases (115). Current models presume that oxygen concentration may directly regulate their activity.

Pharmacological studies demonstrate that the level of certain oxygen metabolites, such as superoxide or other reactive oxygen species (ROS) also regulate HIF-1 activity in tissue culture cells. Mitochondria electron transfer chain (ETC) and NAD(P)H oxidase generate most of the ROS and are suspected to be oxygen sensors (reviewed in (118, 142)). It is not yet known how many oxygen sensors exist nor how they modify HIF activity.

Identification of the Fe(II)- and 2-oxoglutarate-dependent dioxygenases as direct oxygen sensors provides the protein targets to design specific inhibitors of prolyl or asparaginyl hydroxylases. Inhibition of the activities of these enzymes should activate HIF and enhance the hypoxic responses, which may reduce the damage caused by many ischemia-related diseases. Known inhibitors of the prolyl-4-hydroxylase, such as dimethyl-oxalylglycine, have been shown to inhibit HIF-1α degradation at normoxia (109). In a recent study, a constitutively active HIF-1α (the ODD domain was removed) was expressed in mouse epidermis. In the epidermal tissue of these animals, VEGF expression increased 13 fold, and dermal capillaries increased 66% (143). The hypervascular structure developed in those animals did not cause the edema, vascular leakage and inflammation that are common in mice overexpressing VEGF. HIF regulates target genes other than VEGF, and together they are required for building a better vasculature. On the other hand, HIF-1α is overexpressed in many human cancers (144). Vascularization and the grade of human gliomas are correlated with HIF-1α expression level (145). By inhibiting hypoxic responses in cancer cells, researchers may be able to limit tumor vascularization and retard tumor growth.

DISSERTATION ORGANIZATION

Our research group and others have reported that the *C. elegans* genome encodes 5 bHLH-PAS proteins (57, 87, 146). In this dissertation, I initiate functional studies of *aha-1* and *hif-1*. I also describe the expression of the other two bHLH-PAS genes, *cky-1* and T01D3.2.

In Chapter 1, literature related to the study is reviewed. Major topics include: (*i*). An introduction to the genetic model organism *C. elegans*, with emphasis on the recent advancement in *C. elegans* functional genomics and its impact on researchers in the worm community. (*ii*). The structure and function of bHLH-PAS transcription factors. The ARNT subfamily is used to illustrate the structure and functions of bHLH-PAS proteins. I describe the developmental functions of mammalian and *Drosophila* ARNT homologs, with the emphasis on their ability to dimerize with multiple bHLH-PAS proteins. (*iii*). Hypoxia signaling pathway. I describe the current model for hypoxia signaling pathway, also the developmental and pathological functions hypoxia-inducible factor -1.

Chapter 2 is a copy of a research paper published in *Proc. Natl. Acad. Sci. USA*, 2001, 98(14):7916-7921. The title is "The *Caenorhabditis elegans hif-1* gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia". Main findings include: (*i*). *C. elegans* F38A6.3/*hif-1* encodes a homolog of mammalian HIFα. (*ii*). The expression of *C. elegans* HIF-1:GFP fusion protein is regulated by oxygen concentrations. (*iii*). *hif-1* mutants are not able to adapt to hypoxia. (*iv*). *In vitro* expressed HIF-1 and AHA-1 can be co-immunoprecipitated by an antibody specific to AHA-1. (*v*). Efficient AHA-1 nuclear localization in intestinal cells is dependent upon *hif-1* function. Other researchers in the laboratory were also involved in this study. Jo Anne Powell-Coffman was the principal investigator. Rong Guo was a technician for the laboratory. She generated the AHA-1 specific monoclonal antibody 10H8. She also built the *hif-1:gfp* construct pR19 and the HIF-1 expression vector pR33.

In Chapter 3, I summarize the genetic analyses of *aha-1*. The title is "An essential function for *C. elegans* bHLH-PAS proteins: Evidence that *aha-1* and *cky-1* have a role in pharyngeal function". The *aha-1* mutants are larval lethal with feeding defects. Analyses of animals mosaic for *aha-1* expression suggest that *aha-1* has an essential role in pharynx

function. We also demonstrate that *C. elegans* CKY-1, encoded by C15C8.2, interacts with AHA-1 *in vitro*. CKY-1 may represent a new evolutionarily conserved bHLH-PAS subfamily that forms heterodimers with ARNT. A model for AHA-1/CKY-1 heterodimer function in *C. elegans* pharynx is discussed. Other contributors to this work include Shu Wu, who performed the *cky-1* RNAi experiments and AHA-1 homodimer DNA binding assays. Colleen Cary produced the 6His-AHA-1 fusion protein used to generate the AHA-1-specific polyclonal antibody. Jo Anne Powell-Coffman was the principal investigator, and she isolated the *aha-1(ct383)* allele as a postdoctoral fellow in Dr. William Wood's laboratory.

In Chapter 4, I summarize and discuss my thesis research, with emphasis on our study's impact and contribution to various research fields. In brief, the identification of *C. elegans hif-1* as the homolog of mammalian HIFα established *C. elegans* as a simple genetic system to study hypoxic response. AHA-1 has multiple functions in *C. elegans* development, including *ahr-1*, *hif-1* (hypoxia) signaling, and at least one other independent essential function. *C. elegans* CKY-1 likely represents a new bHLH-PAS subfamily that interacts with members of the ARNT subfamily.

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CHAPTER 2

The *Caenorhabditis elegans hif-1* gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia

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Abstract

Hypoxia-inducible factor, a heterodimeric transcription complex, regulates cellular and systemic responses to low oxygen levels (hypoxia) during normal mammalian development or tumor progression. Here, we present evidence that a similar complex mediates response to hypoxia in *Caenorhabditis elegans*. This complex consists of HIF-1 and AHA-1, which are encoded by *C. elegans* homologs of the hypoxia-inducible factor (HIF) α and β subunits, respectively. *hif-1* mutants exhibit no severe defects under standard laboratory conditions, but they are unable to adapt to hypoxia. Although wild-type animals can survive and reproduce in 1% oxygen, the majority of *hif-1*-defective animals die in these conditions. We show that the expression of a HIF-1:green fluorescent protein fusion protein is induced by hypoxia and is subsequently reduced upon reoxygenation. Both *hif-1* and *aha-1* are expressed in most cell types, and the gene products can be coimmunoprecipitated. We conclude that the mechanisms of hypoxia signaling are likely conserved among metazoans. Additionally, we find that nuclear localization of AHA-1 is disrupted in an *hif-1* mutant. This finding suggests that heterodimerization may be a prerequisite for efficient nuclear translocation of AHA-1.

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Introduction

Mammals use both systemic and cellular strategies to adapt to decreased oxygen levels during normal development and homeostasis. Hypoxic tissues secrete growth factors to increase vascularization, and individual cells increase anaerobic metabolism to sustain basic cellular functions (1). Hypoxia also plays a central role in tumor biology, as a mass of cancerous cells must adapt to hypoxia and induce angiogenesis to grow and metastasize (2).

The majority of the transcriptional responses to hypoxia are mediated by hypoxia-inducible factor (HIF) complexes, which consist of α and β subunits. The HIFβ subunit is also termed ARNT (aryl hydrocarbon receptor nuclear translocator) (3-7). When cellular oxygen levels are high, the von Hippel-Lindau tumor suppressor protein (VHL) binds directly to the α subunit and targets it for ubiquitination and proteosomal degradation. However, in hypoxic conditions, degradation of HIFα is inhibited (8-11). This permits HIFα to translocate to the nucleus, dimerize with ARNT, and activate the expression of target genes, which act to increase oxygen delivery or implement metabolic adaptation to hypoxia (2). Despite intensive study, the mechanisms by which cellular oxygen levels are sensed are not well understood. Progress in this field has been limited by the lack of genetic approaches to this important problem.

Widely divergent organisms have the ability to adapt to variable oxygen concentrations, which suggests that mechanisms of hypoxic sensing and response may have been established early in evolutionary history. Here, we investigate the molecular mechanisms of hypoxia response in a powerful genetic model organism, the nematode *Caenorhabditis elegans*. The natural habitat of *C. elegans* is often hypoxic, and *C. elegans* can adapt to very low environmental oxygen levels (12). We find that the *C. elegans hif-1* and *aha-1* gene products form a complex that is similar to the mammalian hypoxia-inducible factor, and we find that *hif-1* function is required for adaptation to hypoxic conditions.

Methods

***C. elegans* Culture.** *C. elegans* were propagated and maintained as described in ref. 13. To isolate the *ia04* mutation, N2 worms were mutagenized with ethyl methyl sulfonate, and the second generation of self-progeny was screened for *hif-1* deletion mutations by PCR (14).

The isolated deletion mutant was backcrossed to Bristol strain N2 nine times. To score viability in hypoxic conditions, animals were cultured on NGM plates in a sealed Plexiglas chamber with constant gas flow at 22°C. Compressed air and pure N2 were mixed to achieve the appropriate oxygen level, which was monitored by an oxygen analyzer. The transgenic *C. elegans* described here were generated by using standard protocols, and in each case, the coinjection marker was *rol-6(su1006)* (15).

Molecular Biology. The complementary DNAs for T01D3.2 and C15C8.2 were isolated from an embryonic cDNA expression library (16). The *hif-1* cDNA plasmid pR8 was excised from the phage clone yk339c5 (from Y. Kohara, National Institute of Genetics, Shizuoka, Japan). The cDNA was modified to include a Kozak consensus translational start site (17) by amplifying the *hif-1* cDNA fragment by using two primers: PASB6f, 5'-GCTCTAGAGCCA-ACCATGGAAGACAATCGGAAAAGAAAC; and PASB2a, 5'-CAGTTGAAACTTTTGA-ATGAACCAGG. The PCR product was then cut with Xba I to generate a 150-bp fragment, which was subsequently cloned into the two Xba I sites of pR8 to create pR33. To generate the *hif-1:GFP* expression construct pR19, a 9.0-kb Kpn I fragment containing the *hif-1* transcriptional start was excised from cosmid F38A6 and cloned into the KpnI site of pSP72. A Pst I-Sma I fragment from the resulting plasmid was cloned into pPD95.75 (provided by A. Fire, Carnegie Institution of Washington). pHJ06 contains all of the *hif-1* coding sequence fused in-frame to green fluorescent protein (GFP). pHJ06 was generated by inserting an *hif-1* cDNA fragment into pR19. To create the *aha-1:gfp* plasmid pHJ02, 2.8 kb of 5' regulatory sequence and the entire coding region of *aha-1* was inserted into the pPD95.75 vector.

Antibodies, in Situ Staining, and Immunoprecipitation. A bacterially expressed fusion protein containing AHA-1 (amino acids 8-57 and 353-451) and glutathione S-transferase was used to immunize mice and generate the 10H8 mAb. Immunostaining was performed as described (18). Images were captured with a Spot RT digital camera (Roche Diagnostics) on a Nikon E800 microscope. A commercially available GFP-specific mAb (Roche Diagnostics) was used to detect HIF-1:GFP on protein blots.

HIF-1 and AHA-1 were expressed in rabbit reticulocyte lysates (Promega TNT system) by using expression constructs pR33 and pJ343 (19). Radiolabeled methionine was included in the translation reaction, and the products were assayed by SDS/PAGE. A rabbit

polyclonal antibody specific to AHA-1 (H.J., S. Wu, R.G., W. B. Wood, and J.A.P.-C., unpublished data) was used to immunoprecipitate AHA-1 and associated proteins. This process was performed as described in ref. 20, with an additional wash in 0.5 M LiCl/100 mM Tris·HCl, pH 7.4.

Results

Identification of hif-1. To determine whether the molecular mechanisms that regulate hypoxia response in *C. elegans* are similar to those described in mammals, we searched the *C. elegans* genome (21) for sequences encoding potential homologs of mammalian hypoxia-inducible factor α subunits (HIF-1α, HIF-2α, and HIF-3α). The proteins that dimerize to form the hypoxia-inducible factor complex are members of a family of transcription factors containing basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) domains (3, 22). The bHLH domain is required for DNA binding and dimerization (23), and the PAS domain mediates interactions with other proteins and regulates dimerization specificity (23, 24). We isolated and sequenced complementary DNAs for the five genes in the *C. elegans* genome that were predicted to contain both of these motifs (*ahr-1*, *aha-1*, F38A6.3, T01D3.2, and C15C8.2). F38A6.3 encodes the *C. elegans* gene product with the highest sequence similarity to the bHLH and PAS domains of mammalian HIF-1α, and we named the gene *hif-1*. The predicted *hif-1* gene product has 42% identity and 67% similarity to human HIF-1α in the bHLH domain, and it has 30% identity and 55% similarity to HIF-1α in the two PAS core domains (Fig. 1a). *C. elegans* HIF-1 is similarly related to the mammalian HIF-2α and HIF-3α proteins within the bHLH-PAS regions. Phylogenetic analysis suggests that *hif-1* diverged from the ancestral HIFα gene before the gene duplication events that gave rise to mammalian HIF-1α, HIF-2α, and HIF-3α.

We designed experiments to test the hypothesis that *hif-1* had biochemical and functional similarities with mammalian HIFα subunits. This hypothesis predicted that (*i*) *hif-1* mutants would be deficient in hypoxia response; (*ii*) the *hif-1* gene product would interact with *aha-1*, the *C. elegans* ortholog of mammalian ARNT (19); (*iii*) HIF-1 protein would be more highly expressed in hypoxic conditions; and (*iv*) both *aha-1* and *hif-1* would be expressed in most, if not all, cells.

hif-1 Mediates Response to Hypoxia. To determine whether *hif-1* function was required for adaptation to hypoxia, we mutagenized *C. elegans* with ethyl methane sulfonate and screened for deletions in the *hif-1* gene. The *hif-1* (*ia04*) mutation is a 1,231-bp deletion of the second, third, and fourth exons. We analyzed *hif-1* complementary DNA in the mutant and determined that the first exon splices to the fifth exon, resulting in deletion of most of the bHLH and PAS domains, a shift in frame, and a premature translational stop (Fig. 1b). This mutation likely results in complete loss of *hif-1* function.

C. elegans adapt to environmental oxygen levels below 4% by decreasing their metabolic rate. At 1% oxygen, their metabolic rate is decreased by half (12). As shown in Table 1, wild-type *C. elegans* embryos hatch and grow to become fertile adults in 1% oxygen. However, 66% of *hif-1* mutants do not survive embryogenesis when cultured in these hypoxic conditions. The requirement for *hif-1* is alleviated when oxygen levels are raised to 2% (Table 1), and there is no critical developmental requirement for *hif-1* when the animals are incubated in standard laboratory conditions (21% oxygen).

Table 1. *hif-1* mutants have reduced viability in hypoxic conditions

	Wild type			*hif-1(ia04)*		
Oxygen levels, %	Embryonic lethality, %	Larval lethality, %	N	Embryonic lethality, %	Larval lethality, %	N
21	1.3	0.8%	475	0.2%	2%	501
2	ND	ND	---	2.3%	1.6%	736
1	3.6	2.4%	503	66.3%	9.4%	596

ND: not determined.

HIF-1 Binds AHA-1. The mammalian hypoxia-inducible factors consist of two protein subunits: HIF-1α (or the related HIF-2α or HIF-3α) and ARNT (or ARNT2 or ARNT3) (2). To determine whether *C. elegans* HIF-1 forms a similar complex, we assayed its ability to bind AHA-1, the ortholog of mammalian ARNT (19). We expressed both gene products in rabbit reticulocyte lysates and immunoprecipitated the proteins with an antibody that specifically recognizes AHA-1. When HIF-1 is coincubated with AHA-1, both proteins are immunoprecipitated (Fig. 2, lane 5). Thus, HIF-1 binds AHA-1 *in vitro*.

HIF-1:GFP Protein Levels Are Increased by Hypoxia. The degradation of mammalian HIFα protein is inhibited when oxygen levels are low. Therefore, we predicted that the expression of *C. elegans* HIF-1 protein would increase in hypoxic conditions. To test this, we generated transgenic animals carrying pHJ06, a reporter gene containing 2.7 kb of *hif-1* 5' regulatory sequence and the entire *hif-1* coding sequence fused to GFP (25). Transgenic worms were incubated in hypoxic conditions for 8 h. A fraction of the worms was then reoxygenated for 10 min. The expression of HIF-1:GFP was assayed by immunoblot by using a GFP-specific antibody. As shown in Fig. 3, HIF-1:GFP expression is increased by hypoxia treatment, and the protein is rapidly degraded upon reoxygenation. *hif-1* mRNA is not substantially increased by hypoxia treatment (data not shown). The levels of AHA-1 protein remained constant, regardless of oxygen concentration (Fig. 3).

aha-1 and hif-1 Are Broadly Expressed. *C. elegans* do not have a complex circulatory system. Instead, they rely on diffusion for gas exchange. Therefore, individual cells must sense and respond to hypoxic conditions. We expected that most, if not all, cells would express *hif-1* and *aha-1*. To test this hypothesis, we assayed the expression of reporter constructs in which *hif-1* or *aha-1* regulatory sequence directed the transcription of GFP. The *hif-1:gfp* fusion gene encoded by the pHJ06 plasmid was not appropriate for this study because it includes the region(s) of *hif-1* that mediate oxygen-dependent degradation. Instead, we used the pR19 plasmid, which contains 2.7 kb of *hif-1* 5' regulatory sequence and the genomic sequence encoding the first 45 aa of *hif-1* fused to GFP. Transgenic animals carrying this reporter express GFP at high levels in every somatic cell (Fig. 4a). The *aha-1:gfp* fusion gene contains 2.8 kb of *aha-1* 5' regulatory sequence and the complete *aha-1* coding region. *aha-1:gfp* is expressed in many cell types throughout development, including hypodermal cells, intestinal cells, pharyngeal cells, and neurons (Fig. 4c). To further analyze AHA-1 expression, we generated the 10H8 mAb, which recognizes AHA-1 on protein blots and *in situ*. 10H8 recognizes AHA-1 in every cell of the *C. elegans* embryo (data not shown). Thus, *aha-1* and *hif-1* are both expressed in most, if not all, cells.

Nuclear Localization of AHA-1 Depends on hif-1 Function. We used the 10H8 antibody to compare the subcellular localization of AHA-1 in wild-type and *hif-1*-defective animals. We reasoned that the interactions between HIF-1 and AHA-1 might be more readily detectable in

cells that did not express other bHLH-PAS proteins, which might dimerize with AHA-1 and/or HIF-1. Therefore, we focused on the intestinal cells, which do not express any of the other *C. elegans* genes that include bHLH and PAS motifs (H.J., S. Wu, R.G., W. B. Wood, and J.A.P.-C., unpublished data). In the intestinal cells of wild-type animals, AHA-1 localization is predominantly nuclear (Fig. 5b). In the *hif-1* mutants, this expression pattern is disrupted. In most cases, the staining is either slightly stronger in the nucleus or diffuse throughout the entire intestine (Fig. 5 d and f). These data suggest that AHA-1 is not efficiently localized to the intestinal nuclei in the *hif-1* mutant. It is also possible that AHA-1 may be destabilized in the absence of *hif-1*. This requirement for *hif-1* is cell type-specific, as AHA-1 is localized to the nuclei of some neuronal cells in the *hif-1* mutant (data not shown). Total AHA-1 levels are equivalent in wild type and *hif-1* mutant animals (Fig. 5a).

Discussion

These data demonstrate that *C. elegans hif-1* is a functional homolog of mammalian HIFα. First, *hif-1* mutants cannot adapt to 1% oxygen. Second, the level of a HIF-1:GFP fusion protein is regulated by oxygen concentration. Third, HIF-1 forms a complex with AHA-1, the *C. elegans* ortholog of ARNT (HIF-1) *in vitro*. The gene products also interact *in vivo*, as the nuclear localization of AHA-1 depends on *hif-1* function in intestinal cells. Fourth, HIF-1:GFP and AHA-1 are expressed in most, if not all, cells.

Physiological Role of hif-1. Although mammalian HIF-1 has an essential role in embryonic development, *hif-1* defective *C. elegans* are viable and fertile when cultured in standard laboratory conditions. This reflects the relatively simple physiology of *C. elegans*. A mammalian embryo relies on hypoxia-induced angiogenesis to oxygenate tissues. Hif-1$^{-/-}$ mice die by E9.0 with severe vascular defects (4, 5). In contrast, an adult *C. elegans* hermaphrodite has no apparent need for specialized respiratory structures or a complex circulatory system. Any cell in the organism is only a few cell widths from the outer surface of the worm or the intestinal lumen (26).

Oxygen sensing and *hif-1* function is likely to be very important in the soil environment inhabited naturally by *C. elegans*. In the laboratory, *C. elegans* are cultured on top of an agar-based medium, and the ambient oxygen concentrations are relatively high.

However, high concentrations of bacteria, the *C. elegans* food source, can deplete oxygen in a soil microenvironment. The nematodes must be able to sense and adapt to these hypoxic conditions.

Regulation of AHA-1 Nuclear Translocation. We find that AHA-1 translocation to the nuclei of intestinal cells is inefficient in *hif-1* mutants. This result was not predicted by the prevalent models for hypoxia signaling. In the mammalian cell lines commonly used to study hypoxia-inducible factor or aryl hydrocarbon receptor (AHR) signaling, ARNT is localized to the nucleus constitutively. After HIF-1 or activated AHR translocates to the nucleus, it forms a dimer with ARNT (2). However, in the *Drosophila* embryo, *Drosophila* ARNT (encoded by the *tango* gene) apparently remains cytoplasmic until a bHLH-PAS dimerization partner is expressed (27, 28). We conclude that the role of AHA-1 in the formation and nuclear localization of an active transcriptional complex may depend on cell type-specific factors, such as the expression of other bHLH-PAS proteins. We will explore this hypothesis by examining the expression of other bHLH-PAS genes in those cells that localize AHA-1 to the nucleus in the absence of *hif-1*.

Importance of a Genetic Model System for the Study of Hypoxia. A thorough understanding of the molecular mechanisms by which cells sense and respond to hypoxia is essential to the treatment of many human diseases, including cancer, stroke, and cardiac failure. However, several critical questions remain. The oxygen sensors are undiscovered, and it will be important to determine how they regulate HIFα stability. Insulin signaling has also been shown to activate the hypoxia-inducible factor (29-31), but the roles of this interaction during hypoxia response, cellular differentiation, or tumor progression are not understood. Other signal transduction pathways may also modify hypoxia-inducible factor to modulate cellular response to low oxygen levels (32).

C. elegans is an increasingly powerful model system for the dissection of signal transduction pathways, and further genetic analysis of *hif-1* and its interacting genes will likely reveal mechanisms of hypoxia signaling and response that are conserved throughout the animal kingdom. In support of this hypothesis, a putative homolog of VHL has recently been identified in *C. elegans* (33), and the *cul-2* gene encodes another evolutionarily conserved component of the ubiquitin-proteasome proteolytic mechanism (34).

While this manuscript was in review, two studies were published that demonstrated that mammalian HIFα is targeted for VHL-mediated degradation by proline hydroxylation (35, 36). The modified proline and an essential leucine have been identified in HIFα, and these residues are conserved in *C. elegans* HIF-1 (35).

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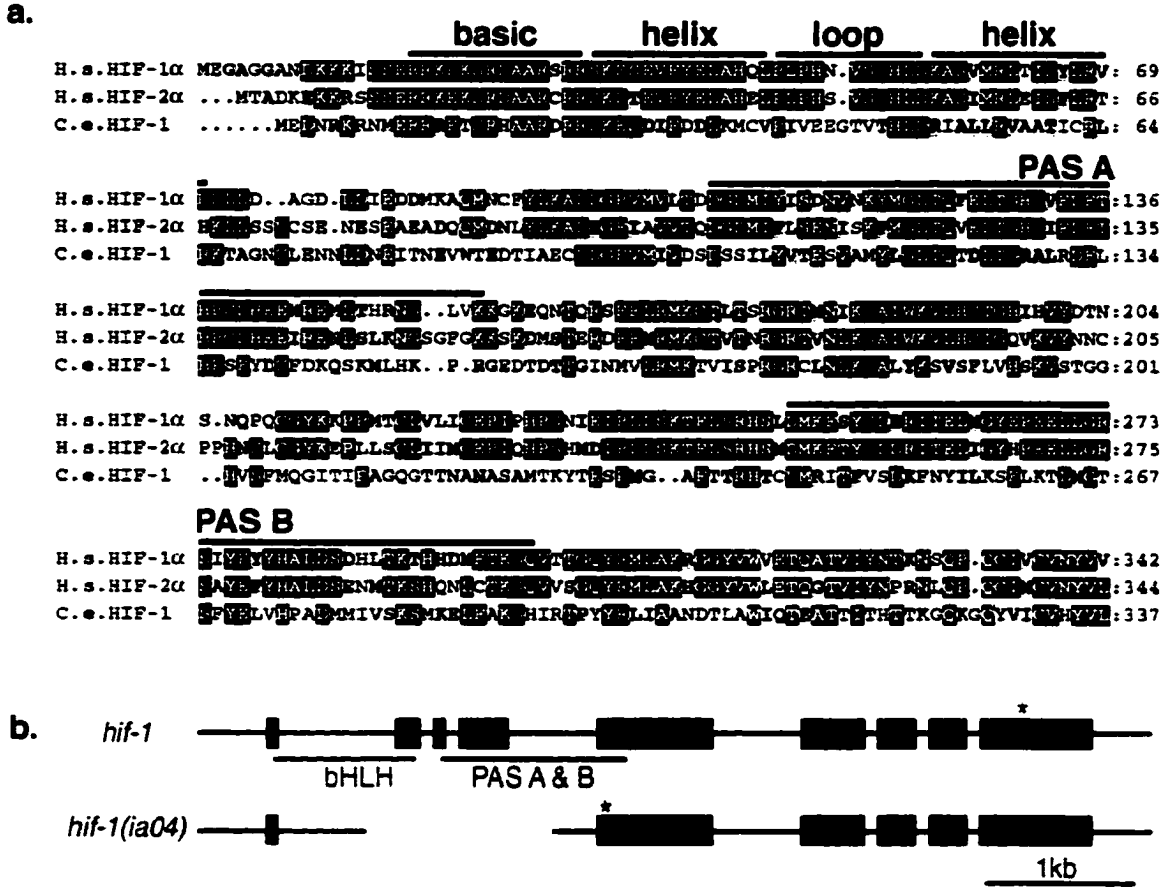

Fig. 1. (**a**) Conserved N-terminal domains of the predicted HIF-1 protein, and the *hif-1* genomic structure. Alignment of the N-terminal amino acid sequences of human HIF-1α (H.s. HIF-1α; U22431), HIF-2α (H.s. HIF-2α; U81983), and *C. elegans* HIF-1 (C.e. HIF-1: AF364604). Identical and similar amino acids are highlighted in black and gray, respectively. (**b**) Genomic structure of *hif-1* and the *hif-1(ia04)* deletion mutant. Boxes represent exons. * indicate the first stop codon in the *hif-1* ORF. The *ia04* mutation is a 1,231-bp deletion of the second, third, and fourth exons. This introduces a frameshift and premature stop in the mutant mRNA.

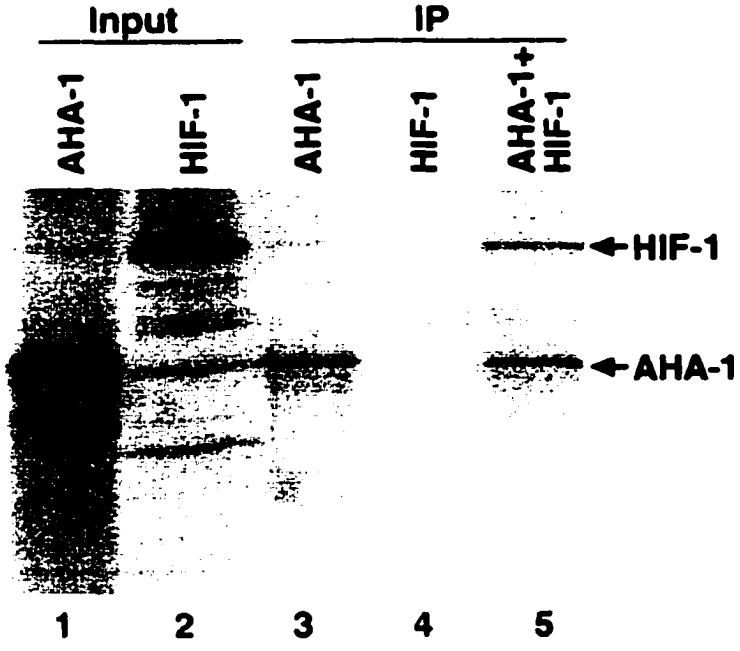

Fig. 2. HIF-1 binds AHA-1. HIF-1 and AHA-1 were expressed in rabbit reticulocyte lysates in the presence of [^{35}S]methionine (lanes 1 and 2), subjected to immunoprecipitation with an AHA-1-specific antibody (IP; lanes 3-5), fractionated by SDS/PAGE. The antibody immunoprecipitates AHA-1 (lane 3) but not HIF-1 (lane 4). When AHA-1 and HIF-1 are coincubated, the antibody pulls down both proteins (lane 5). The molar ratio of HIF-1/AHA-1 in lane 5 is 1:2.5.

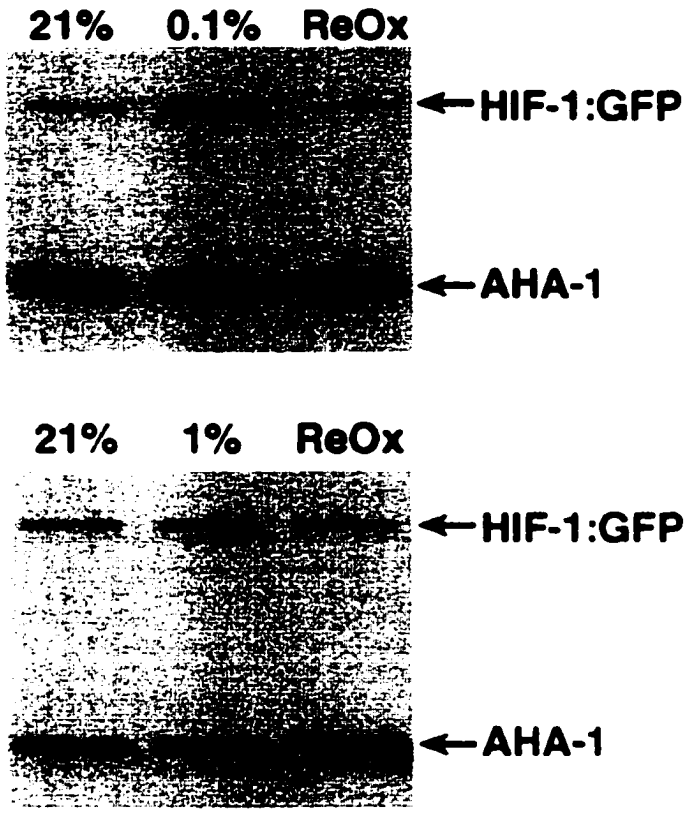

Fig. 3. HIF-1:GFP protein levels are increased by hypoxia. The transgenic *C. elegans* strain pHJ06 *Ex6*, which expresses the complete HIF-1 protein fused to GFP, was harvested before (21% O2) and after incubation in 0.1% oxygen (Upper) or 1% oxygen (Lower) for 8 h. In each experiment, a fraction of the hypoxia-treated worms were reoxygenated for 10 min (ReOx). Worms were lysed by boiling in loading buffer, and equal amounts of total protein were loaded in each lane. The expression of HIF-1:GFP and AHA-1 was assayed by probing the immunoblot with a GFP-specific antibody and the AHA-1-specific mAb 10H8.

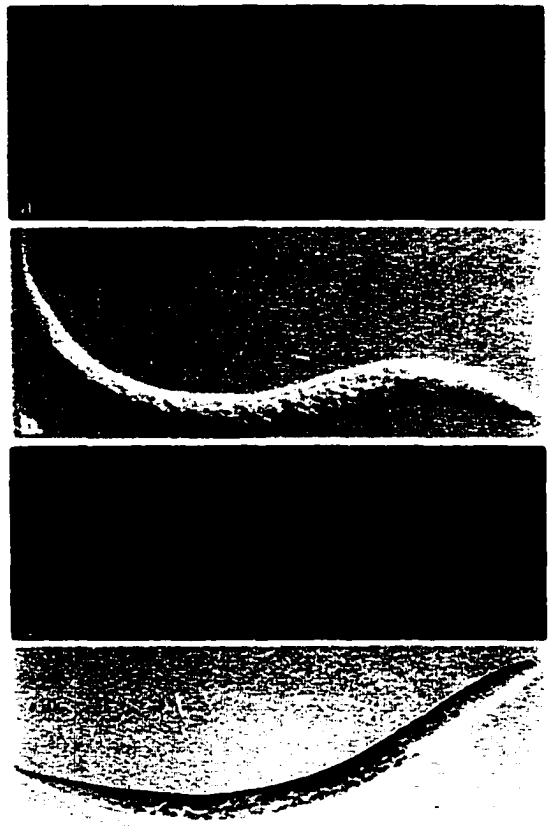

Fig. 4. *hif-1* and *aha-1* promoters direct expression of GFP reporter genes in most cell types. Fluorescent images and Nomarski images of a *C. elegans* first-stage larvae expressing *hif-1:gfp* (a and b) and *aha-1:gfp* (c and d).

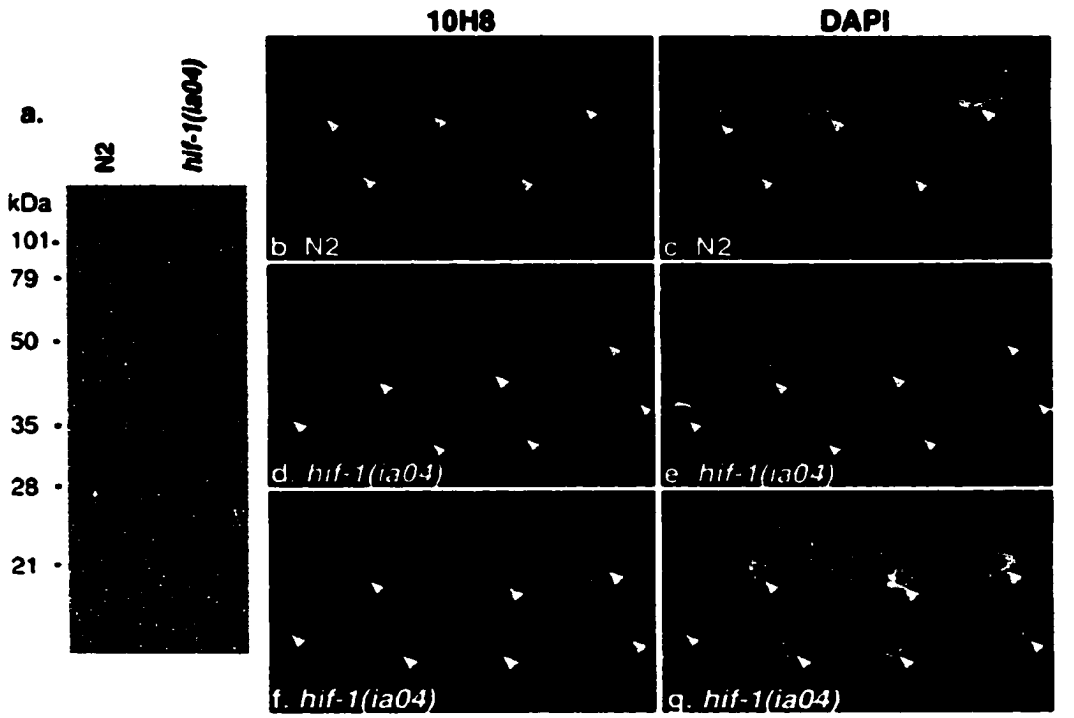

Fig. 5. Subcellular localization of AHA-1 is disrupted in the *hif-1*(*ia04*) mutant. (a) Equal amounts of total protein from wild-type (N2) or *hif-1*(*ia04*) were size-fractionated by SDS/PAGE, blotted, and probed with the AHA-1-specific antibody 10H8. (b, d, and f) Immunostaining of intestinal cells with 10H8 in wild-type (N2) (b) or *hif-1* mutant adults (d and f). (c, e, and g) The 4',6-diamidino-2-phenylindole staining of the cells that are shown in b, d, and f, respectively. The arrowheads label the positions of intestinal cell nuclei.

CHAPTER 3

An essential function for *C. elegans* bHLH-PAS proteins: Evidence that *aha-1* and *cky-1* have a role in pharyngeal function

A paper to be submitted to *Developmental Biology*

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ABSTRACT

The family of basic-helix-loop-helix-PAS (bHLH-PAS) transcription factors mediate diverse cellular processes, including several important cell fate decisions during development and adaptations to environmental signals. Molecular and biochemical data indicate that *C. elegans aha-1* encodes the ortholog of the mammalian aryl hydrocarbon receptor nuclear translocator (ARNT). Previous studies have shown that AHA-1 is expressed broadly, and it can form complexes with AHR or HIF-1. To better understand the role of *aha-1* in *ahr-1* and *hif-1* signaling and to reveal other functions of bHLH-PAS proteins, we isolated deletion mutations that abolish *aha-1* function. *aha-1* deletion mutants arrest as larvae and are defective in feeding. The larval arrest phenotype of *aha-1* mutants is much more severe than the *ahr-1*, *hif-1* double mutants (which are viable), suggesting that *aha-1* has additional functions. We analyzed the expression patterns of the two remaining bHLH-PAS genes, T01D3.2 and *cky-1*. A T01D3.2:GFP reporter is expressed in two interneurons. *cky-1* may represent a new bHLH-PAS gene subfamily, and a CKY-1:GFP reporter is expressed mainly in the pharynx, which is the feeding organ of the worm. These data suggest that AHA-1 and CKY-1 might interact and function in the pharynx. Several lines of evidence support this model: AHA-1 and CKY-1 form a DNA-binding complex *in vitro*. AHA-1 is nuclear localized in the subset of pharyngeal cells that also express CKY-1:GFP. A *cky-1:aha-1* fusion, in which a *cky-1* upstream sequence was placed upstream of the *aha-1* coding

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[2] S. W. is a graduate student in the laboratory who performed the gel-shift assay for AHA-1 homodimer.

[3] C. C. is an undergraduate assistant who generated the 6 His-AHA-1 fusion protein.

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sequence, was able to rescue the *aha-1* mutant phenotype. We propose that *aha-1* has an essential function in the *C. elegans* pharynx.

INTRODUCTION

During the development of multicellular organisms, individual cells must recognize and respond to developmental and environmental signals. Many important cellular responses, such as cell differentiation, proliferation, apoptosis and migration, require specific changes in gene expression. To understand these processes, we must first identify the transcriptional factors involved and determine how they are regulated. The family of transcription factors containing basic, helix-loop-helix, and Per-ARNT-Sim (PAS) domains (bHLH-PAS proteins) regulate several very important developmental processes and mediate adaptation to certain environmental changes, including circadian rhythm regulation, neuronal differentiation and adaptation to hypoxia (reviewed in Crews and Fan, 1999 and Gu *et al.*, 2000).

One of the most studied bHLH-PAS protein complexes is mammalian AHR and its nuclear translocator ARNT. AHR is a ligand-activated receptor, and it mediates the toxic effects of certain pollutants, including dioxin and polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (Bock, 1994). Mammalian HIF-1α also interacts with ARNT. The heterodimer is called hypoxia-inducible factor-1 (HIF-1), and it regulates transcriptional responses to hypoxia (Wang *et al.*, 1995). Although AHR, HIF-1α and most of the other bHLH-PAS proteins are each dedicated to a specific developmental or cellular function, proteins in the ARNT subfamily are included in every DNA-binding bHLH-PAS protein complexes identified thus far. This range of protein interactions predicts that ARNT proteins should be required for multiple developmental functions, and this has been confirmed by genetic analyses. For example, ARNT-deficient mice have defects in embryonic angiogenesis that are similar to those exhibited by HIF-1α null mice (Iyer *et al.*, 1998; Kozak *et al.*, 1997; Maltepe *et al.*, 1997; Ryan *et al.*, 1998). Mice lacking ARNT2 or SIM1 have similar defects in neuronal differentiation in the hypothalamus (Hosoya *et al.*, 2001; Keith *et al.*, 2001; Michaud *et al.*, 2000; Michaud *et al.*, 1998). Both MOP3/ARNT3 and CLOCK null mice are arrhythmic (Antoch *et al.*, 1997; Bunger *et al.*, 2000; King *et al.*, 1997). *Drosophila* ARNT is

encoded by the *Tango* (*tgo*) gene. The *tgo* mutant phenotypes also confirmed that it has multiple functions in *Drosophila* development. These including CNS midline specification (Tgo/Sim), tracheal pit formation (Tgo/Trh) and development of distal antenna, distal leg and bristles (Tgo/Ss)(Emmons *et al.*, 1999; Sonnenfeld *et al.*, 1997).

Biochemical analyses have shown that ARNT homodimers bind a class B E-box enhancer that contains two adjacent ARNT half sites (Antonsson *et al.*, 1995; Sogawa *et al.*, 1995; Swanson *et al.*, 1995). Further, this enhancer can direct reporter gene expression in mammalian tissue culture cells in an ARNT-dependent manner (Antonsson *et al.*, 1995; Sogawa *et al.*, 1995). However, the putative transcriptional targets of ARNT homodimers have not been identified. The role of ARNT homodimers is also unclear in genetic model systems. Efficient nuclear localization of *Drosophila* Tgo is dependent upon co-expression of a heterodimerization partner (Ward *et al.*, 1998). Similarly, the ARNT homolog in *C. elegans*, AHA-1, is not efficiently nuclear localized in intestinal cells in the absence of its dimerization partner HIF-1 (HIFα homolog) (Jiang *et al.*, 2001). This data suggests that if homodimers form *in vivo*, they are not translocated to or stabilized in the nucleus as efficiently as bHLH-PAS heterodimeric complexes.

Other studies suggest that ARNT function may be regulated by other cell type specific signal transduction pathways. Mammalian ARNT is phosphorylated at multiple sites (Levine and Perdew, 2001). Dephophorylation of ARNT reduces its activity to form heterodimers with AHR *in vitro* (Berghard *et al.*, 1993). Protein Kinase C (PKC)-dependent phosphorylation events have been demonstrated to enhance AHR signaling and ARNT homodimer activity (Chen and Tukey, 1996; Long *et al.*, 1999). However, it is not clear whether PKC directly phosphorylates ARNT and/or AHR.

C. elegans is a simple genetic system, and it is ideal to study the complex function and regulation of bHLH-PAS proteins. The *C. elegans* genome encodes 5 bHLH-PAS genes (Jiang *et al.*, 2001; Ledent and Vervoort, 2001). These include *aha-1*, *ahr-1* and *hif-1*, which are homologs of ARNT, AHR and HIFα respectively (Jiang *et al.*, 2001; Powell-Coffman *et al.*, 1998). *C. elegans* AHR-1, AHA-1 and HIF-1 have been shown to be biochemically similar to their mammalian cognates. *C. elegans hif-1* mutants fail to adapt to hypoxia (Jiang *et al.*, 2001). To clarify the role of *aha-1* in *ahr-1* and *hif-1* signaling in *C. elegans* and to

identify other potential functions of bHLH-PAS proteins, we isolated deletion mutations in *aha-1*. We report that *aha-1* has an essential function during larval development, in addition to its roles in AHR and hypoxia signaling. The two remaining bHLH-PAS genes, *cky-1* and T01D3.2 are expressed in the pharynx and a pair of interneurons, respectively. We present evidence that AHA-1 interacts with CKY-1. Further, expression of *aha-1* from the *cky-1* upstream fragment is sufficient to rescue *aha-1* mutants to viability.

MATERIALS AND METHODS

Worm Strains, Transformation and RNAi

The following *C. elegans* strains were used in this study: wild type, Bristol N2; CB1091, *unc-13(e1091)* I; MT1196, *lin-11(n566)* I; DR466, *him-5(e1490)* V; *mut-2(r459)* I. ZG3, *aha-1(ct383) mut-2(r459)* I; ZG5 *aha-1(ia01)/lin-11(n566)* I; ZG6 *aha-1(ia02)/lin-11(n566)* I.

Worm cultures were grown under standard conditions (Brenner, 1974). DNA constructs were transformed into worms by co-microinjection with the roller marker pRF4, *rol-6(su1006)* (Mello *et al.*, 1991). Multiple lines for each transformation were generated and analyzed.

Isolation of aha-1 Mutations

The ZG3 strain contains both the *aha-1(ct383) Tc1* insertion and the *mut-2(r459)* allele, which allows transposon mobility. PCR screening protocols (Plasterk, 1995) were used to identify the *ct383* allele in a transposon insertion library, which was constructed in the laboratory of J. Priess (U. of Washington). A PCR-based sib selection protocol was used to identify the two *aha-1* deletion alleles, *ia01* and *ia02*, among ZG3 progeny (Plasterk, 1995). The isolated deletion alleles were out-crossed with wild type up to 6 times. The closely linked marker *unc-13(e1091)* was crossed on and off both alleles to eliminate other potential mutations and to remove the *mut-2(r459)* prior to phenotypic analysis. Both alleles were balanced with *lin-11(n566)*.

Phenotypic Analysis of aha-1 Mutants

The larval lethality phenotype of *aha-1* mutants was assayed using ZG5 *aha-1(ia01)/lin-11(n566)* and ZG6 *aha-1(ia02)/lin-11(n566)*. The number of arrested larvae were counted 2 days after hatching when the smaller arrested mutant larvae were readily distinguishable from their wild type siblings.

The feeding efficiency assay was modified from the methods originally described (Avery and Horvitz, 1987) (Avery and Horvitz, 1990) as follows. For wild type N2, synchronized larvae were assayed at the first and second larval stages (most of the *aha-1* mutants arrest at these stages). The progeny of *aha-1(ia01)/lin-11(n566)* were assayed 24, 36 and 48 hours after hatching. 24 hours after hatching, *aha-1* mutants are readily distinguishable from their bigger wild type siblings. After incubating in a 1:100 mixture of ink (Pelikan, Fount India) and OP50 bacterial suspension for 20 minutes, worms were quickly paralyzed by adding sodium azide to a final concentration of 0.05%. The presence of ink particles in the gut lumen was checked by microscopy. The experiments were repeated 3 times, each time about 30 animals were checked for the presence of ink particles in the gut lumen. To observe the "stuffed" phenotype, 2 day-old *aha-1* mutants were placed on NGM plates spotted with OP50 mixed with 1:100 dilution of ink. After 24 hours, animals were photographed using a Spot RT digital camera (Roche Diagnosis) on a Nikon E800 microscope.

cky-1 and T01D3.2 GFP Reporter Constructs

To create the *cky-1* GFP reporter construct pR15, a 4.3 kb Sal I/Bgl II fragment from cosmid C15C8, containing 2.5 kb sequences upstream of the *cky-1* transcription start and part of the genomic coding region (codons 1-102), was cloned into the Sal I/BamH I sites of pPD95.67.

The T01D3.2 GFP reporter construct pR14 was generated in two steps. First, a 7 kb Kpn I fragment from cosmid T01D3 was subcloned into the Kpn I site of pBluescript SK(II) to generate pR10. Then a 3.8 kb Sma I/Nhe I fragment from pR10, which contains 3.6 kb 5' sequences from the T01D3.2 translational start and part of the genomic coding regions (codons 1-31), was cloned into the Hind III/Xba I (Hind III end was blunted using DNA

polymerase I Klenow) of pPD95.75. The GFP reporter constructs were created by A. Fire and colleagues (Carnegie Institute of Washington).

aha-1 Rescue Constructs

Two *aha-1* rescue constructs were used in this study. (*i*) pHJ28 is a subclone of C25A1 (32,739 bp - end), and it contains the entire *aha-1* genomic region, including 2.8 kb sequences 5' to ATG, the entire coding region and 2.1 kb sequences downstream of the poly A addition site. (*ii*). pHJ32 was designed to ectopically express AHA-1 in a subset of pharyngeal cells. A *cky-1* genomic fragment containing the same 5' sequences used in pR15 (Sal I/Sac I fragment from pR15) was fused to an *aha-1* genomic fragment (BamH I/Bgl II fragment from pHJ28). To complete the *aha-1* coding sequence, an oligonucleotide adapter containing the first 6 codons of *aha-1* was added (Figure 4A).

DNA Binding Assay and Immunoprecipitation

The *cky-1* cDNA clone pR7 was isolated from an embryonic cDNA library (Okkema and Fire, 1994). In pR7', the sequence proceeding the translational start site was modified to create the Kozak consensus site (Kozak, 1987). CKY-1 and AHA-1 were expressed in rabbit reticulocyte lysates (Promega TNT system) using expression vectors pR7' and pJ343 (Powell-Coffman *et al.*, 1998).

Immunoprecipitation assays were performed as described previously (Jiang *et al.*, 2001). Briefly, proteins were translated *in vitro* and labeled with [^{35}S-]Methionine. The AHA-1 specific monoclonal antibody 10H8 (Jiang *et al.*, 2001) was used to immunoprecipitate AHA-1 and associated proteins. The precipitates were assayed by SDS-PAGE.

Gel mobility shift assays were performed as described previously (Powell-Coffman *et al.*, 1998). For the CKY-1/AHA-1 DNA binding assay, the XRE probe was 5'-TCGAGGGGCATT**GCGTG**ACACC, annealed to 5'-TCGAGGTGT**CACGC**AATGCCCC. The XREmut competitor was 5'-TCGAGGGGCATT**GACTG**ACACC, annealed to 5'-TCGAGGTGT**CAGTC**AATGCCCC (the binding site is in bold, and the mutations are underlined). For the AHA-1 homodimer DNA binding assay, the AHAwt probe was 5'-TCGACAAAGGT**CACGTG**ATTGTGG, annealed to 5'-

TCGACCACAAT**CACGTG**ACCTTTG. The AHAmut1 competitor was 5'-TCGACAAAGGT**CAC<u>A</u>TG**ATTGTGG, annealed to 5'-TCGACCACAAT**CA<u>T</u>GTG**ACCTTTG. Another competitor, AHA-1mut2, was 5'-TCGACAAAGGT**CA<u>TA</u>TG**ATTGTGG, annealed to 5'-TCGACCACAAT**CA<u>TA</u>TG**ACCTTTG. The XRE and AHAwt probes were radioactively labeled using DNA polymerase I Klenow and [α-^{32}P]dCTP. For supershift assays, 1µl of rabbit anti-AHA-1 polyclonal antibody or 10H8 was added to the reaction mix. 4E10 is a negative control monoclonal antibody. The AHA-1-specific polyclonal antibody was generated by innoculating rabbits with a fusion protein, in which 6 histidines were fused to amino acids 6-451 of AHA-1. The antibody was further purified from the sera using the Promega Tetralink™ system by using an AHA-1 fusion protein containing a biotin tag. This fusion protein was generated using Promega Pinpoint™ system, in which the same AHA-1 coding region was included. The experiments were performed as recommended by the manufacturer.

RESULTS

aha-1 is Essential

To analyze the function of *C. elegans aha-1*, we isolated deletion mutations in *aha-1* using a transposon-mediated mutagenesis protocol (Plasterk, 1995). We used PCR-based techniques to screen a library of transposon insertions, and isolated *aha-1(ct383)*, which contains a *Tc1* insertion in the 6th intron (cosmid C25A1 35,345 bp, Figure 1B). *aha-1(ct383)* homozygous animals are viable and fertile. Prior studies have shown that *Tc1* insertions in introns do not necessarily impair function (Rushforth *et al.*, 1993). We screened for *aha-1* deletion alleles that were generated from imprecise excision of the transposon. We isolated two *aha-1* deletion alleles, *ia01* and *ia02*. *aha-1(ia01)* contains a 2,447 bp deletion (cosmid C25A1, 34,680-37,127 bp), which removes all the coding sequences between exon 2 and the poly A addition site (Figure 1C). No *aha-1* transcripts were detected in *aha-1(ia01)* mutants (RT-PCR, data not shown). *aha-1(ia02)* contains a 1,408 bp deletion (cosmid C25A1 35,345-36,753 bp) of part of exon 3 and exons 4-6. A 281 bp *Tc1* fragment remains. This results in a frame shift and a premature stop codon immediately downstream of the

deletion (Figure 1D; the structure of the mutant transcript was confirmed by RT-PCR). These molecular analyses suggest that *ia01* and *ia02* are both strong loss-of-function alleles and are likely nulls. The arrest phenotypes for *ia01* and *ia02* are very similar (see below), and except where noted, the *ia01* allele was used for the studies described here.

Animals that are homozygous for either *aha-1* deletion mutation arrest development as larvae, and this defect is completely penetrant (*ia01*, n=814; *ia02*, n=768). 92.5% (n=133) of *ia01* mutants arrest at the first or second larval stage, and the remainder arrest development before adulthood. Some of the arrested larvae survive up to 2 weeks. The arrested larvae have pale intestines. This phenotype is characteristic of starved animals, and it suggested that *aha-1* mutants might have pharyngeal or intestinal defects.

The pharynx is a rhythmically contracting neuromuscular organ at the anterior end of the animal that pumps the bacterial food source into the intestine and initiates digestion. To assay pharyngeal function in *aha-1* mutants, we mixed ink with the bacterial food source. After feeding for 20 minutes, ink particles are visible in the intestine of >97% of first or second stage wild type larvae (Figure 2A&C). In contrast, 24 hours after hatching, less than 50% of the *aha-1* mutants contain ink particles in the intestines (Figure 2C). This phenotype becomes more severe after longer periods of arrest (Figure 2C). In addition, 35% (n=94) of the terminally arrested larvae had a "stuffed" pharynx phenotype 3-4 days after hatching. In these animals, bacteria are lodged in the pharyngeal lumen (Figure 2B). The severity of the *aha-1* mutant phenotype demonstrates that *aha-1* has essential developmental functions that are independent of *ahr-1* or *hif-1* function, *ahr-1*, *hif-1* single or double mutants are viable (Jiang *et al.*, 2001; unpublished data).

The larval arrest phenotype of *aha-1(ia01)* was fully rescued by pHJ28, a subclone of C25A1 that contains the *aha-1* genomic region (see Materials and Methods for details).

Expression of C. elegans bHLH-PAS Genes cky-1 and T01D3.2

To better understand the essential functions of *aha-1*, we examined the expression pattern of potential bHLH-PAS dimerization partners. The *C. elegans* genome encodes 5 members of this family. *ahr-1*, *hif-1*, and *aha-1* have been described in previous studies (Jiang *et al.*, 2001; Powell-Coffman *et al.*, 1998). *ahr-1* is expressed primarily in neurons

(Qin and Powell-Coffman, unpublished data). *hif-1* is ubiquitously expressed and is required for adaptation for hypoxia (Jiang *et al.*, 2001). We generated GFP reporter constructs to examine the expression patterns of the two remaining bHLH-PAS genes, T01D3.2 and C15C8.2/*cky-1*.

The T01D3.2:GFP reporter pR14 contains 3.6 kb upstream sequence and codons 1-31 of T01D3.2 fused to GFP. The positions of the neuronal cell bodies and axon tracts are shown in Figures 3C&D. In mid-embryogenesis, GFP expression is observed in 4 cells (2 each side) in the head. At the 1.5-fold embryonic stage, two GFP-expressing cells (one on each side) begin neuronal differentiation. The other two undergo apoptosis (Figure 3A&B). Postembryonic GFP expression is only detected in two interneurons. We conclude that the cells expressing T01D3.2:GFP are the AVH interneurons (Sulston *et al.*, 1983; White *et al.*, 1986). No essential function has been described for these neurons.

The CKY-1:GFP reporter pR15 contains 2.5 kb upstream sequence and codons 1-102 of CKY-1. GFP expression was first detected in the pharyngeal primordium at the two-fold stage of embryogenesis (Figures 4B&C), and pharyngeal expression was maintained during all later developmental stages. A mature pharynx consists of 80 nuclei and 5 different cell types (Albertson and Thomson, 1976). CKY-1:GFP was expressed in all pharyngeal muscle cells, g2 gland cells, epithelial cells, marginal cells and pharyngeal-intestinal valve cells (Figure 5, D-F). CKY-1:GFP expression was not detectable in any of the pharyngeal neurons or in the g1 gland cells. 5-10 cells immediately outside of pharynx also expressed CKY-1:GFP (Figures 4B&D, indicated by asterisks).

AHA-1 is Nuclear Enriched in a Subset of Pharyngeal Cells

Nuclear localization of AHA-1 may require co-expression of a dimerization partner (Jiang *et al.*, 2001). To determine whether expression of CKY-1:GFP correlated with nuclear localization of AHA-1, we stained wild type animals with the AHA-1 specific monoclonal antibody 10H8. As previously reported, AHA-1 is expressed in most, if not all, somatic cells (Jiang *et al.*, 2001). As shown in Figures 5A&B, AHA-1 is also detectable in oocyte nuclei. In the larval pharynx, AHA-1 is nuclear enriched in the pharyngeal muscle cells, epithelial cells, structural marginal cells and pharyngeal intestinal valve cells (Figures 5C&D).

However, AHA-1 nuclear enrichment is not observed in any of the 20 pharyngeal neurons. This expression pattern suggests that AHA-1 has a transcriptional function that is specific to a subset of pharyngeal cells. Further, it suggests that a dimerization partner might be co-expressed in these cells.

CKY-1 Forms a Heterodimer with AHA-1 in vitro

Nuclear localization of AHA-1 in the pharyngeal cells that express CKY-1:GFP suggests that AHA-1 and CKY-1 may interact. We tested this hypothesis by coimmunoprecipitation experiments. We expressed both gene products in rabbit reticulocyte lysates and immunoprecipitated the proteins with the AHA-1 specific monoclonal antibody 10H8. As shown in Figure 6B, 10H8 is able to precipitate AHA-1, but not CKY-1. When CKY-1 is coincubated with AHA-1, both proteins are immunoprecipitated (Figure 6B, lane 5).

We used the TBLASTN algorithm (Altschul *et al.*, 1990) to search Genebank for potential homologs of *cky-1*. We identified 3 potential mammalian *cky-1* genes: a predicted human Open reading frame (ORF), XM_100570 and two murine expressed sequence tags (EST), BB623011 and BB594112, which were originally isolated from cDNA libraries prepared from brain tissues. The alignment of the bHLH domains is shown in Figure 6A. The basic and helix-loop-helix domain of CKY-1 shares 58% identity and 72% similarity with this potential human homolog.

To determine whether CKY-1 and AHA-1 could form a DNA-binding complex, we performed electrophoretic mobility shift assays (EMSA). The bHLH domains of CKY-1 and its homologs are most similar with that of the AHR family (Ledent and Vervoort, 2001) (Figure 6A), and AHR-1 and AHA-1 interact to bind the xenobiotic response element (XRE, sequence 5' -TGCGTG). When AHA-1 and CKY-1 are coincubated, they bind the XRE probe and decrease its mobility (Figure 6C, lane 4). The binding is sequence specific: the labeled probe can be displaced by a 200-fold excess of unlabeled XRE (Figure 6C, lane 6) but not by an unlabeled competitor containing a mutant XRE (XREmut2, 5' -TGCGTG mutated to 5' -TGACTG) (Figure 6C, lane 7). Addition of the AHA-1 specific monoclonal

antibody generated a supershift (SS). (Figure 6C, lane 8). The formation of only one supershift band suggests that only one AHA-1 molecule was present in the complex.

To determine whether AHA-1 could form a homodimeric DNA binding complex, we performed a similar EMSA assay using *in vitro* expressed AHA-1. As shown in Figure 6D, AHA-1 was able to bind to a probe containing a class B E-box (5'-CACGTG). The addition of 10H8 generates two supershift bands, which indicates that the complex contains two AHA-1 molecules. The negative control monoclonal does not cause the complex to decrease mobility.

cky-1:aha-1 is able to Rescue aha-1(ia01) Mutants

The genetic and biochemical data presented so far support two non-exclusive models: AHA-1 may function as a dimer with CKY-1 in the pharynx. Alternatively, AHA-1 homodimers may have an essential function in the pharynx or in other tissues. If formation of an AHA-1/CKY-1 heterodimer is essential for pharyngeal function, then *cky-1* loss-of-function mutations should cause lethality. We attempted to decrease *cky-1* function via double-stranded RNA-mediated interference (RNAi), and progeny of the treated animals were viable with no obvious defects. However, this negative result may not be informative since some genes and tissues are known to resistant to RNAi in *C. elegans* (Tavernarakis *et al.*, 2000; Timmons *et al.*, 2001). In general, RNAi experiments are more likely to impair gene function for *C. elegans* genes with embryonic functions than those with postembryonic phenotypes (Maine, 2001). Indeed, worms treated with *aha-1* dsRNA are also viable (Fraser *et al.*, 2000).

As a second approach, we generated animals that were mosaic for *aha-1* function. To create these animals, we generated a chimeric construct, pHJ32 (Figure 4A), in which a *cky-1* upstream fragment directs the expression of the *aha-1* transcript. Extrachromosomal arrays of this construct were able to fully rescue the larval arrest phenotype of *aha-1* deletion mutants. To verify the AHA-1 expression pattern in these animals, the rescued animals were stained with the AHA-1 specific antibody 10H8, and the detectable staining was mostly restricted to the pharyngeal tissue (Figure 4G) and two tail neurons (a pair of PHA or PHB phasmid

neurons, data not show). Thus, AHA-1 is required in a subset of cells which is defined by CKY-1:GFP expression.

DISCUSSION

C. elegans CKY-1 Form a Heterodimer with AHA-1 in vitro

In addition to *aha-1*, the *C. elegans* genome encodes 4 other bHLH-PAS genes (Jiang *et al.*, 2001; Ledent and Vervoort, 2001). In prior studies, we have shown that AHR-1 and HIF-1 can interact with AHA-1 *in vitro* (Jiang *et al.*, 2001; Powell-Coffman *et al.*, 1998). Since the larval arrest phenotype of *aha-1* mutants is much more severe than that of *hif-1*, *ahr-1* double mutants (the *hif-1*, *ahr-1* double mutants are viable (unpublished data)) and known DNA-binding bHLH-PAS complexes all contain members of the ARNT subfamily, we examined other *C. elegans* bHLH-PAS proteins for potential dimerization partners of *aha-1*. *In vitro* immunoprecipitation and gel-shift assays demonstrate that CKY-1 forms DNA-binding heterodimers with AHA-1. *In vitro* expressed CKY-1 is able to co-immunoprecipitate with AHA-1 (Figure 6B, lane 5) and CKY-1/AHA-1 heterodimers bind to DNA probe containing the xenobiotic response element (XRE) (Figure 6C). The attempts to express the only other *C. elegans* bHLH-PAS protein, T01D3.2, were unsuccessful, and whether it interacts with AHA-1 is unclear.

We further identified several mammalian homologs of *cky-1* in sequence databases. The two murine EST (expressed sequence tag) homologs were originally isolated from cDNA libraries prepared from brain tissues. The predicted protein sequences of these genes contain a bHLH domain that is highly homologous to CKY-1 (Figure 6A). Given the strong conservation of AHA-1 function to its mammalian ortholog ARNT (Jiang *et al.*, 2001; Powell-Coffman *et al.*, 1998), we predict that the mammalian CKY-1 proteins form heterodimers with ARNT to execute their functions. CKY-1 may represent a new bHLH-PAS protein family that is able to dimerize with ARNT.

Evidences for an Essential Function of aha-1 in the Pharynx

Animals homozygous for either *aha-1* deletion alleles arrest at larval stages. Phenotypic analyses of the *aha-1* mutant animals revealed a possible function in the pharynx.

The *aha-1* mutant animals appear starved, and 35% of the terminally arrested mutants have a "stuffed" pharynx phenotype (Figure 2B). The *C. elegans* pharynx is a feeding organ that grinds the bacterial food source, transfers the food into the intestine and initiates digestion. Consistent with the possible pharyngeal function for *aha-1*, ink-feeding assays reveal that *aha-1* mutants feed poorly (Figure 2C). Furthermore, expression of *aha-1* from the *cky-1* upstream fragment (pHJ32, Figure 4A) is sufficient to rescue the *aha-1* larval arrest phenotype. 10H8 immunostaining of the rescued animals revealed that AHA-1 is mainly detected in the pharynx (Figure 4G) and a pair of phasmid neurons (data not shown). Thus we propose that *aha-1* has an essential function in the pharynx and the *aha-1* larval arrest phenotype most likely resulted from the feeding defects. However, a yet uncharacterized pharynx-related *aha-1* function may also be essential for larval development.

The completely penetrant larval arrest phenotype of *aha-1* mutants is striking. Most of the previously described mutations that impair pharyngeal function are viable. Many genes that regulate pharyngeal function have been identified in genetic screens for mutations that impair feeding but are not lethal, including a number of *eat* (eating-defective) and *phm* (pharyngeal muscle defective) mutations (Avery, 1993). The *aha-1* mutants arrest at larval stages, so it is likely to be missed in that screen. After examining the genetic map of the mutations isolated in that screen, we conclude that *aha-1* is indeed not included. Other feeding defective mutations have also been identified, such as *eat-20* (Shibata *et al.*, 2000) and *ceh-22* (Okkema and Fire, 1994). Mutant animals for them are not lethal (The *ceh-22* mutants are partially lethal, the homozygous *ceh-22* mutants can be maintained as a very weak strain) (Okkema and Fire, 1994).

Complete inability to feed does result in lethality, as evidenced by mutations in *pha-1*, *pha-2* and *pha-4* (Avery, 1993; Granato *et al.*, 1994; Mango *et al.*, 1994). In *pha-4* mutants, the pharynx primordium is not formed (Mango *et al.*, 1994). In *pha-1* mutants, the pharynx primordium form but they fail to undergo terminal differentiation (Granato *et al.*, 1994). Both *pha-1* and *pha-4* mutants are embryonic lethal. In *pha-3* mutants, the pharynxes are misshapen, and most of the mutants arrest as the first stage larvae (Avery, 1993). In contrast, the pharynxes of *aha-1* larvae do not have major structural defects when observed by microscopy. MYO-2 immunostaining using monoclonal antibody 9.2.1 (Epstein *et al.*,

1982) indicates that pharyngeal myosin expression and structure are normal in the *aha-1* mutant animals (data not shown). We also stained the mutant animals with monoclonal antibody MH27, which recognizes an adherens junction protein present around hypodermal cells in the pharynx (Francis and Waterston, 1991; Labouesse, 1997), and the pharyngeal expression pattern is comparable to that of wild type N2 (data not shown). We conclude that *aha-1* mutants do not have dramatic structural defects in the pharynx.

C. elegans feeding is accomplished by two separately controlled pharynx muscle motions, isthmus peristalsis and pumping. Isthmus peristalsis transfer the bacterial food source from the anterior corpus to the terminal bulb through isthmus. When pharyngeal neuron M4 is laser ablated, isthmus peristalsis is defective and the bacteria are lodged in the anterior lumen, resulting in a "stuffed" phenotype (Avery and Horvitz, 1987). Since the bacterial can not reach the terminal bulb and intestine, the larvae arrest. The terminally arrested *aha-1* mutants also have a "stuffed" pharynx phenotype (Figure 2B). However, in most of the cases, bacteria are lodged in the entire pharynx. Furthermore, the fusion construct *cky-1:aha-1* is not expressed in the pharyngeal neurons (data not shown), yet it still rescued the *aha-1* larval lethal phenotype. *aha-1* is unlikely involved in M4-regulated isthmus peristalsis.

The *C. elegans kel-1* encodes the homolog of *Drosophila Kelch* (Ohmachi *et al.*, 1999). The *kel-1* mutants are feeding-defective and arrest as early larvae. But the pharynx structure of the *kel-1* mutants is normal. Overall, *aha-1* and *kel-1* mutants have similar phenotypes. However, KEL-1 is expressed in the pharyngeal g1 gland cells and is required for feeding in these cells (Ohmachi *et al.*, 1999). It is unlikely that the larval arrest of *aha-1* mutants are caused by g1 cell defects since CKY-1:AHA-1 is not expressed in g1 gland cells (data not shown), yet the fusion is still able to efficiently rescue the *aha-1* mutant phenotype.

C. elegans pharyngeal development is organized by the *fork head* transcription factor PHA-4 (Horner *et al.*, 1998; Kalb *et al.*, 1998). Besides its role in early pharynx primordium development, *pha-4* function is also required for viability after the pharynx is formed (Gaudet and Mango, 2002; Mango *et al.*, 1994). When *pha-4* temperature sensitive mutants (*pha-4(ts)*) were shifted to the restrictive temperature at mid-embryogenesis, they hatched

and arrested as first stage larvae, with a "stuffed" pharynx phenotype similar with that observed in the terminally arrested *aha-1* mutants (Gaudet and Mango, 2002) (Figure 2B). This data suggests that both PHA-4 and AHA-1 have required functions in the pharynx in late embryogenesis and/or larval development. PHA-4 and AHA-1 may have similar transcriptional targets, or bHLH-PAS proteins may regulate a subset of the genes that are ultimately controlled by PHA-4. PHA-4 has been demonstrated to directly mediate most or all pharyngeal genes expression by binding to the TRTTKRY element in the promoter of these genes (Gaudet and Mango, 2002). Analysis of *cky-1* upstream sequence identified 5 potential PHA-4 binding sites within 1 kb upstream of ATG, suggesting that PHA-4 may regulate *cky-1* expression directly.

Two non-exclusive models can be used to explain the AHA-1 function in the pharynx: AHA-1/CKY-1 heterodimer may have an essential pharyngeal function. Alternatively, AHA-1 homodimers may have a critical role in the pharynx. If the heterodimers are the only bHLH-PAS gene function required in the pharynx, we predict that *cky-1* mutants will have similar defects as *aha-1* mutants, which is larval arrest. Treatment of wild type animals with *cky-1* dsRNA did not result in a mutant phenotype (data not shown). Interestingly, feeding wild type animal with *aha-1* dsRNA also did not result in larval lethal phenotype (Fraser *et al.*, 2000). Thus, these negative results may not be informative. The resolution of this issue will require mutational analysis of *cky-1*.

However, expression of *aha-1* in a subset of pharyngeal cells using a *cky-1:aha-1* fusion construct rescues the *aha-1* larval arrest phenotype, which provides strong support for the heterodimer model. In addition, analysis of AHA-1 immunostaining revealed that AHA-1 is nuclear localized in the subset of pharyngeal cells that also express CKY-1:GFP, but not in the pharyngeal neurons which do not express CKY-1:GFP (data not shown). Like *Drosophila* Tgo, AHA-1 lacks the N-terminal nuclear localization signal (NLS) of mammalian ARNT, its efficient nuclear localization in intestinal cells requires the presence of its dimerization partner (Jiang *et al.*, 2001). That suggests that AHA-1 and CKY-1 may interact in those pharyngeal cells.

Maternally Contributed AHA-1 and AHA-1 Homodimer

AHA-1 *in situ* immunostaining reveals that AHA-1 is detected in the developing oocyte nuclei, suggesting that AHA-1 is maternally contributed. However maternally contributed AHA-1 is unlikely have an essential function in *C. elegans* development. 10H8 immunostaining of the *cky-1:aha-1* rescued animals demonstrated that the CKY-1:AHA-1 is not expressed in the germline or intestine (some of the maternally contributed proteins are expressed in the intestine and subsequently transferred into oocyte) (data not shown). AHA-1 expression is not detected until 2-fold embryonic stage. Yet zygotic AHA-1 expression from this fusion construct is still fully capable of rescuing the *aha-1* mutants.

AHA-1 can form a homodimer *in vitro*. However, at least in some *C. elegans* cell types, co-expression of a dimerization partner is required for efficient nuclear localization of AHA-1. In *hif-1* mutants, AHA-1 is not efficiently nuclear localized in the intestinal nuclei (Jiang *et al.*, 2001). However, low levels of AHA-1 homodimers in the intestinal nuclei may be sufficient to execute developmental functions. The *cky-1:aha-1* rescued worms will allow for further examination of the possible function of *aha-1* homodimers by carefully analyzing other potential phenotypes of the rescued animals.

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FIGURE LEGENDS

Figure 1. *aha-1* genomic structure and its mutant alleles. **A.** *aha-1* corresponds to the genomic sequence for C25A1.11. The exons are represented by black boxes. **B.** *aha-1(ct383)* is a *Tc1* insertion allele that contains a *Tc1* transposon in the 6th intron. The *Tc1* insertion is indicated by a reverse triangle. **C** and **D.** *aha-1* deletion alleles isolated as described in

materials and methods. *ia01* is a 2.4 kb deletion which deleted most of the *aha-1* coding region. *ia02* is a 1.4 kb deletion which deleted part of exon 3, exon 4-6, also retained part of *Tc1* sequence (281 bp *Tc1* fragment is indicated by gray box). The start and stop codon of the transcripts is marked.

Figure 2 Ink feeding assays. **A.** Wild type animals after feeding ink. Ink particles are abundant in the gut lumen. **B.** The "stuffed" pharynx of *aha-1* mutants. Ink and bacteria are lodged in the pharynx and are undetectable in the gut lumen. **C.** Quantitation of the *aha-1* mutant feeding defects. *aha-1* mutants are assayed 24, 36 and 48 hrs after hatching. Data are the average of three independent experiments ± SD.

Figure 3 T01D3.2:GFP reporter expression pattern. **A &B.** In 1.5-fold embryo, T01D3.2:GFP reporter is expressed in 4 cells in the head. Two cells on the left side of the embryo are shown here. One cell is extending an axon (indicated by arrowheads), and the other is round and dying (indicated by arrows). **B.** Normarski image of **A. C.** After hatching, GFP is observed in a pair of neurons (AVHR&L). **D.** Illustration of **C**. GFP-expressing neurons are indicated by black ovals. Axon tracts are represented by thick or dotted lines.

Figure 4 *cky-1* reporter expression pattern. **A.** *cky-1* fusion constructs. pR15 is the *cky-1:gfp* reporter, which contains a Sal I-Bgl II fragment from cosmid C15C8 fused with GFP. In pHJ32, a Sal I-Sac I fragment containing the *cky-1* upstream region is fused with the complete *aha-1* genomic region. Black boxes, *cky-1* exons; green box, GFP coding region; red boxes, *aha-1* exons. **B-F.** *cky-1* reporter pR15 expression pattern. **B.** GFP expression is observed in the pharynx primordium at 2-fold embryonic stage. **D.** In third stage larvae, expression is observed in several cell types in the pharynx including: e1, e2 and e3, epithelial cells; m1-8, muscle cells; vpi, pharyngeal intestinal valve cells; mc, marginal cells and g2, gland cells. **E.** GFP expression pattern in the terminal bulb. **G.** 10H8 *in situ* immunostaining of the *ia01* mutants rescued by pHJ32. **C, F** and **H** are the Normarski images of **B, E** and **G.** Asterisks indicate GFP-expressing cells outside of the pharynx.

Figure 5 Immunolocalization of AHA-1 using the monoclonal antibody 10H8. **A.** AHA-1 is present in the nuclei of a subset of pharyngeal cells. A second stage larva is shown here. The staining cells (indicated by arrows) in this focal plane include e2, epithelial cell; m1, m2, m5-m8, muscle cells; mc, marginal cell and vpi, pharyngeal intestinal valve cell. **C.** AHA-1 expression is also detected in the developing oocyte nuclei. Arrows indicate the positions of oocyte nuclei. **B** and **D.** DAPI staining of **A** and **C.**

Figure 6 AHA-1 forms homodimers and heterodimers with CKY-1. **A.** Alignment of the bHLH domains of *cky-1* and *ahr-1* family. Accession no. are CKY-1, AF370361. AHR-1, AFO39539570. hAHR, *Homo sapiens* AHR, P35869 and mAHR, *Mus musculus* AHR A46266. XM_100570 is a human predicted open reading frame (ORF), BB623011 and BB594112 are two murine ESTs (expressed sequence tags) that are expressed in brain tissues. **B.** CKY-1 binds AHA-1. CKY-1 and AHA-1 were expressed in rabbit reticulocyte lysates in the presence of [^{35}S]methionine (lanes 1 and 2), subjected to immunoprecipitation with an AHA-1-specific antibody (IP, lanes 3-5). The antibody immunoprecipitates AHA-1 (lane 3), but not CKY-1 (lane 4). When AHA-1 and CKY-1 are coincubated, the antibody pulls down both proteins (lane 5). **C** and **D.** Gel mobility-shift assays for DNA binding of the AHA-1:CKY-1 heterodimer and the AHA-1 homodimer. AHA-1 and CKY-1 were expressed independently, and incubated with labeled XRE or AHA-1wt probes. Unlabeled specific competitors were added into some reactions. To create supershifts (SS), 1μl of AHA-1-specific monoclonal antibody 10H8 (m), or polyclonal antibody (p) or a non-specific monoclonal antibody control (C) were added to some reactions. The reaction mixes were analyzed by nondenaturing polyacrylamide gels. Arrows indicate shift bands. NS, non-specific band.

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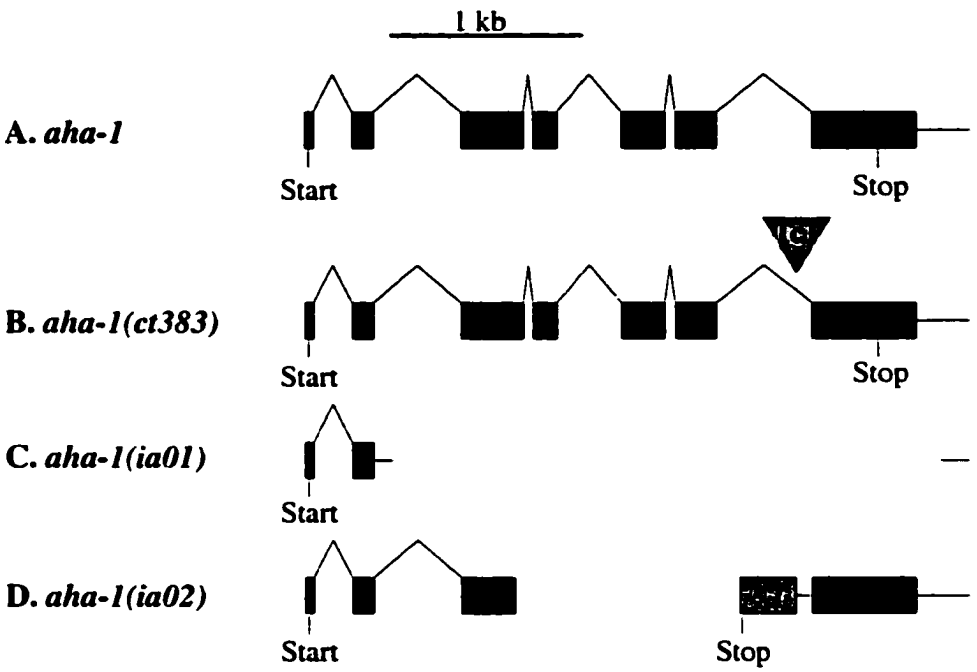
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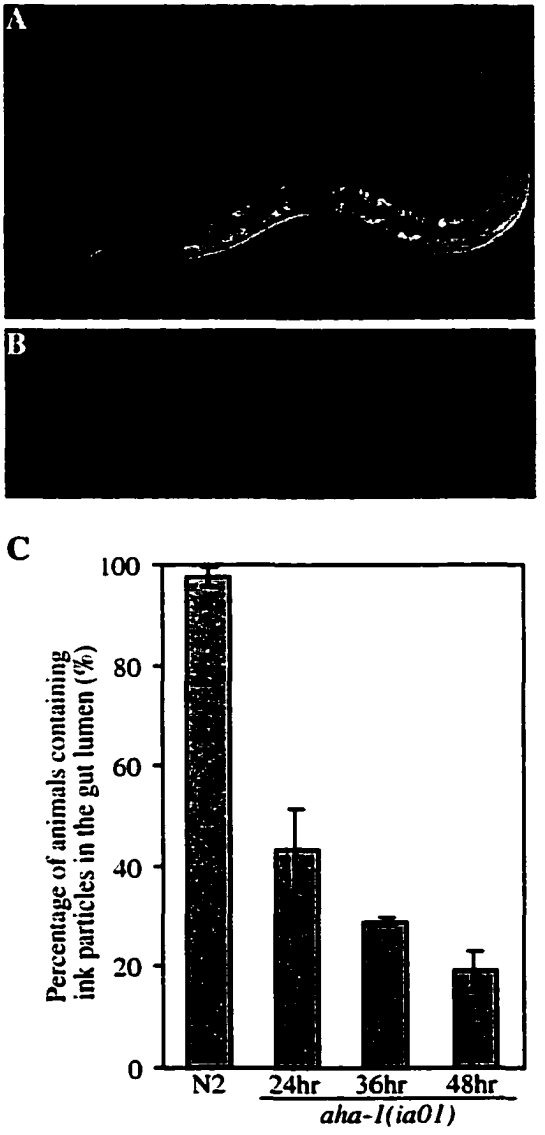
Figure 1


A
B
C
Percentage of animals containing ink particles in the gut lumen (%)
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40
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24hr
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aha-1(ia01)

Figure 2

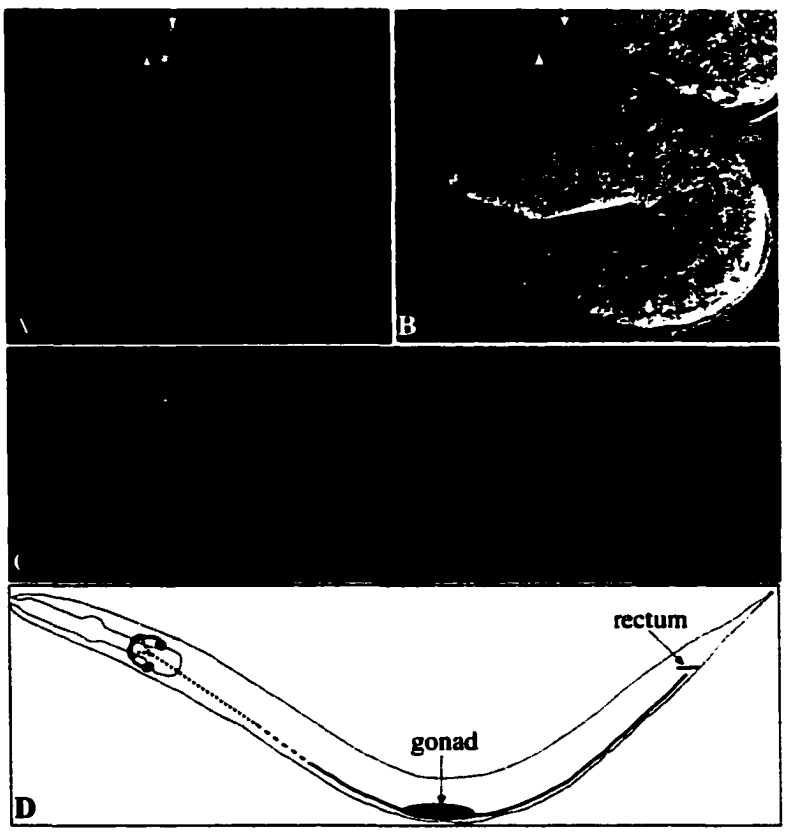

Figure 3

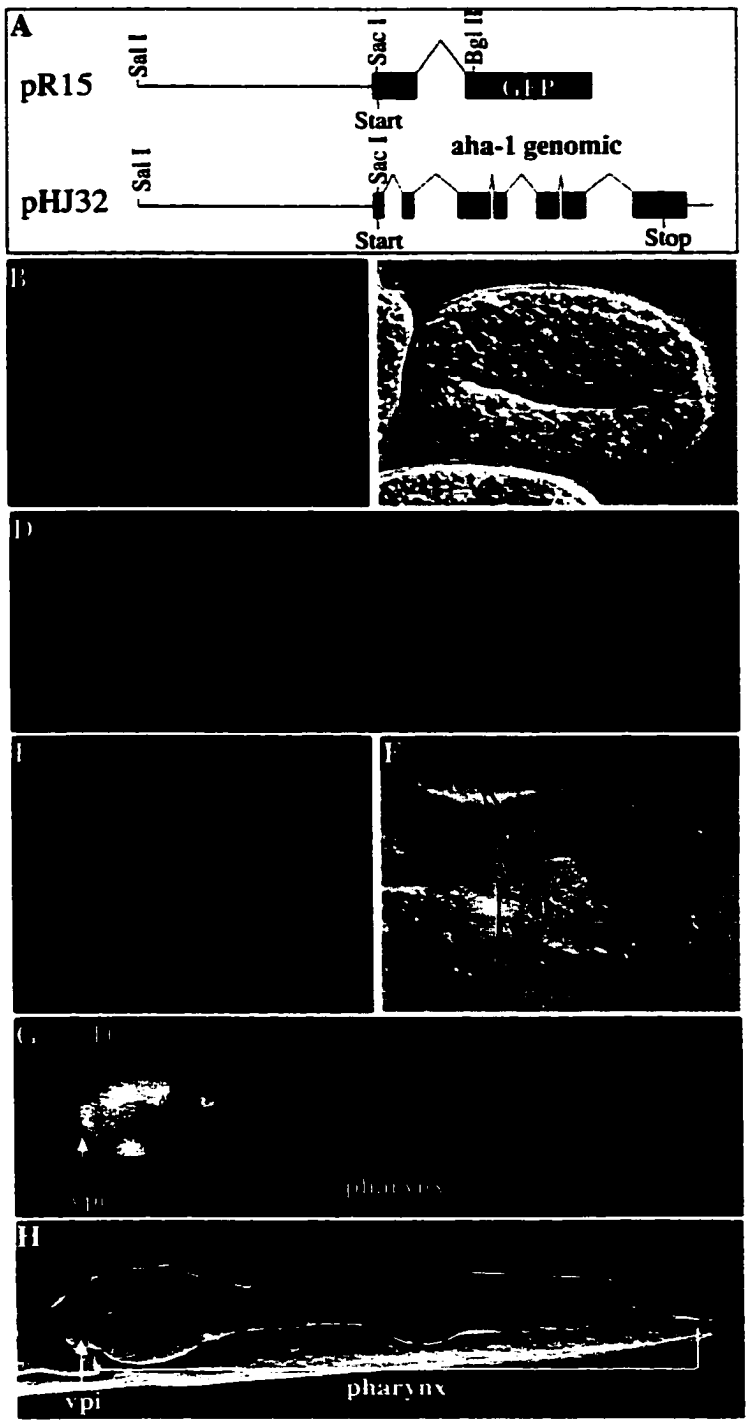
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Figure 4

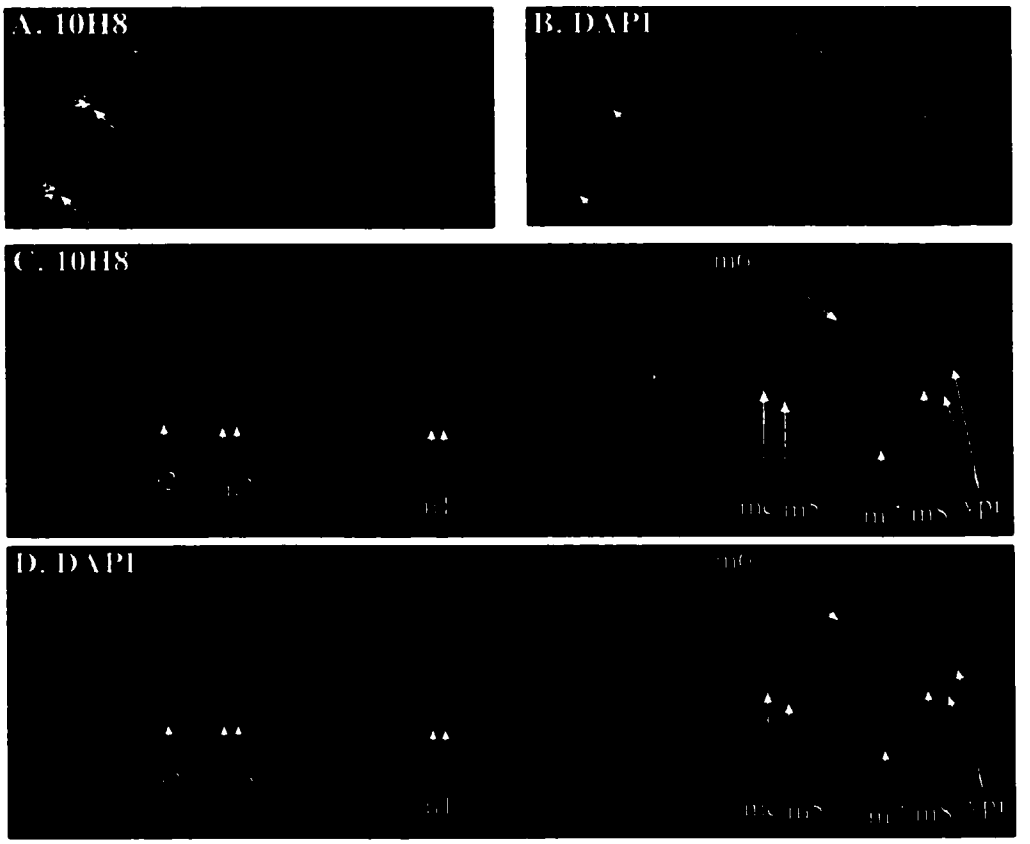
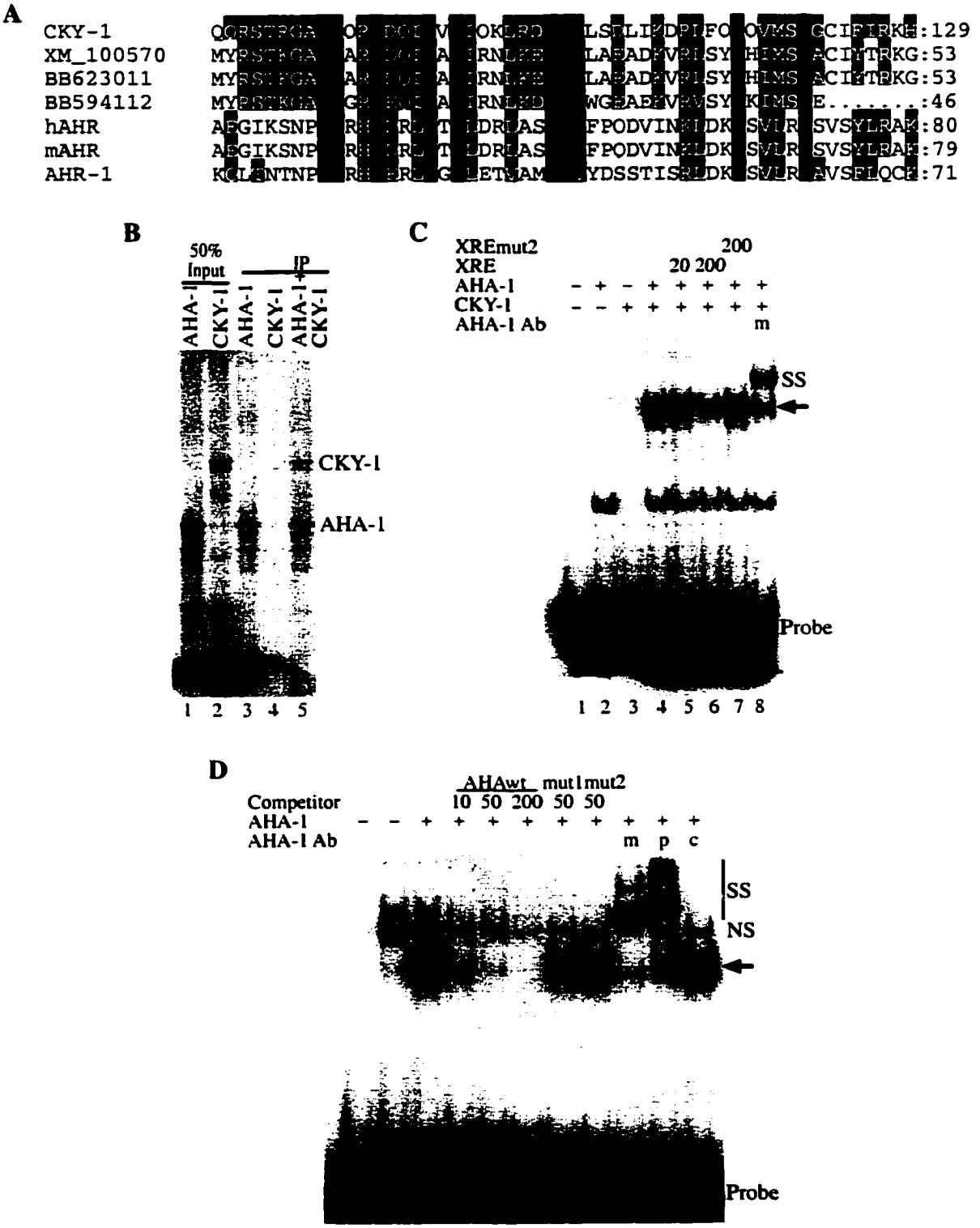


Figure 5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XRE
AHA-1
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1 2 3 4 5 6 7 8
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Figure 6

CHAPTER 4 GENERAL CONCLUSIONS

SUMMARY

In this dissertation, I use both genetic and biochemical methods to analyze the function of bHLH-PAS proteins in *C. elegans*. Major findings include: 1) The *C. elegans* genome encodes 5 bHLH-PAS proteins, and their unique expression patterns are revealed by either GFP/*LacZ* reporters or *in situ* immunostaining. 2) The *C. elegans* bHLH-PAS gene *hif-1* encodes the homolog of mammalian HIFα and is required for adaptation to hypoxia. 3) The *C. elegans* homolog of ARNT, *aha-1*, has an essential function. Phenotypic analyses and expression studies suggest that AHA-1 forms a dimer with CKY-1, and the complex has an essential role in pharyngeal function. 4) *C. elegans* CKY-1 likely represents a new bHLH-PAS subfamily that dimerizes with members of the ARNT subfamily.

The C. elegans genome encodes 5 bHLH-PAS proteins

Blast searches for sequences similar to the mammalian ARNT PAS domain identified 5 bHLH-PAS genes in *C. elegans* (Chapter 2). In a previous study, molecular and biochemical criteria were used to show that two of them, *aha-1* and *ahr-1*, are the orthologs of mammalian AHR and ARNT (1). We generated reporter constructs for all 5 bHLH-PAS genes. *ahr-1* GFP and *LacZ* reporters are expressed in many neurons, and *ahr-1* has a role in neuronal differentiation and migration (Qin and Powell-Coffman, manuscript in preparation). The *aha-1:gfp* reporter pHJ02 is broadly expressed (Chapter 2, Figure 4). This expression pattern is confirmed by immunostaining with the AHA-1-specific monoclonal antibody 10H8 (Chapter 2, Figure 5 and Chapter 3, Figure 5). The pR19 F38A6.3/*hif-1* GFP reporter is ubiquitously expressed in all the somatic cells (Chapter 2, Figure 4). The pR15 C15C8.2/*cky-1* GFP reporter is expressed mainly in the pharynx, starting at the 2-fold embryonic stage and persisting through later developmental stages. It is also expressed in 5-10 cells immediately outside of pharynx (Chapter 3, Figure 4). The pR14 T01D3.2 GFP reporter is expressed in the AVH interneurons. It is also expressed in the AVH sister cells in the embryo, which later undergo apoptosis.

The protein sequences and their expression patterns enable us to formulate testable hypotheses about their functions. These data led to the identification of *hif-1* as the functional homolog of mammalian HIFα (Chapter 2). These studies also identified CKY-1 as a potential new dimerization partner for AHA-1 (Chapter 3), and suggested an essential function for *aha-1* in the pharynx (Chapter 3).

hif-1 encodes the homolog of mammalian HIFα and is required for adaptations to hypoxia in C. elegans

The protein sequence of HIF-1 is most similar with that of the mammalian HIFα subfamily (Chapter 2, Figure 1). The pR19 HIF-1:GFP reporter directs ubiquitously GFP expression (Chapter 2, Figure 4). These data prompted us to hypothesize that *hif-1* is the homolog of mammalian HIFα. We tested this hypothesis using both biochemical and genetic methods. There were three major findings. 1) HIF-1:GFP fusion protein expression is increased in response to hypoxia, and it is quickly degraded upon re-oxygenation (Chapter 2, Figure 3). 2) Animals defective in *hif-1* function fail to adapt to hypoxia. While most progeny of the wild type N2 survive to adulthood (94%) at 1% oxygen, only 24% of the progeny of *hif-1*mutants survive to adulthood (Chapter 2, Table 1). 3) Both *in vitro* and *in vivo* data indicate that HIF-1 forms a heterodimer with AHA-1. *In vitro* expressed HIF-1 is able to co-immunoprecipitate with AHA-1 (Chapter 2, Figure 2). AHA-1 immunostaining reveals that efficient nuclear localization of AHA-1 in intestinal cells is dependent on the function of *hif-1* (Chapter 2, Figure 5). These data demonstrate that *C. elegans hif-1* is the functional homolog of mammalian HIFα.

Identification of *hif-1* as the homolog of mammalian HIFα establishes a simple genetic system to study the complex hypoxia signaling pathway. *C. elegans* is a powerful model system for studying signal transduction pathways. Further genetic analysis of *hif-1* and its interacting genes will likely reveal conserved mechanisms of hypoxia signaling. This is underscored by the results of a recent independent study of *C. elegans hif-1* by Peter Ratcliffe and his colleagues (2). They also identified *hif-1* as the homolog of mammalian HIFα. Furthermore, they identified a *C. elegans* VHL homolog, *vhl-1* and isolated a *vhl-1* mutant. HIF-1 expression is increased in the *vhl-1* mutant in normoxic conditions. More importantly,

HIF-1 expression is also increased in *egl-9* mutants. Thus, genetic analyses of *hif-1* led to the identification of the first direct oxygen sensor, the *C. elegans* EGL-9 prolyl hydroxylase and its mammalian homologs PHDs (prolyl hydroxylase-domain). The strong evolutionary conservation of the hypoxia signaling pathway further testifies to the promise of the *C. elegans* genetics in studying complex pathways.

Hypoxia-inducible factor-1 is involved in the physiopathology of various human diseases (3). Answers to many questions about hypoxia signaling still remain elusive. Are asparaginyl/prolyl hydroxylases the only oxygen sensors for hypoxia responses? How can hypoxia responses be effectively inhibited or enhanced *in vivo*? A more thorough understanding of the hypoxia signaling pathways will enable us to find better treatments for diseases related to hypoxia such as heart attack, stroke and cancer.

To gain insights to these questions, another student in the laboratory, Chuan Shen initiated a study to identify downstream targets of HIF-1 in *C. elegans* using DNA microarray technologies. One goal of the study to is to identify *in vivo* target genes of the *C. elegans* hypoxia-inducible factor complex AHA-1/HIF-1. This will allow the construction of hypoxia-responsive reporter genes that can be used to monitor the activity of hypoxia signaling in *C. elegans*. Additionally, they will enable both traditional forward genetic methods and functional genomics tools (such as systemic RNAi) to identify other regulators of hypoxia signaling.

C. elegans is also a very suitable model organism for drug discoveries (4). *egl-9* was originally identified as a locus that once mutated, confers the resistance to the paralysis caused by *Pseudomonas aeruginosa* (5). Subsequently, EGL-9 was shown to modify HIF-1 in an oxygen-dependent manner (2). The toxicity of *P. aeruginosa* is due in part to hydrogen cyanide (HCN) production (6). It is not yet known whether HIF-1 over-expression is sufficient to protect worms from HCN. If true, this offers an opportunity to use *C. elegans* to screen for chemicals that will also confer resistance to HCN. Those chemicals will be candidates for specific inhibitors of the prolyl hydroxylase involved in hypoxia signaling.

AHA-1 is able to dimerize with multiple bHLH-PAS proteins and CKY-1 may represent a new bHLH-PAS subfamily that interacts with ARNT

Results presented in this dissertation also confirm that AHA-1, like its homologs in mammals and flies, is able to dimerize with multiple bHLH-PAS proteins (Figure 1). AHA-1 interacts with AHR-1 to form a complex that is similar to the mammalian AHR/ARNT heterodimer (1). AHA-1 is also able to form heterodimers with HIF-1 (Chapter 2, Figure 2) and CKY-1 (Chapter 3, Figure 6B&C). T01D3.2 expression is low in rabbit reticulocyte lysates, and this has precluded assays for interaction (CO-IP) with AHA-1. Whether it is able to interact with AHA-1 needs to be further tested. Shu Wu has shown that AHA-1 also forms a homodimer and binds to the class B E-box (Chapter 3, Figure 6).

Consistent with *aha-1*'s role as a common dimerization partner for other bHLH-PAS proteins, *aha-1* mutants exhibit multiple defects. *C. elegans aha-1* has an essential function during larval development (Chapter 3). Arrested larvae also have neuronal defects that are similar to those exhibited by *ahr-1* mutants (Figure 2). It will be interesting to test whether the *cky-1:aha-1* rescued mosaic animals are sensitive to hypoxia treatment as the hypoxia signaling model (*C. elegans* HIF-1/AHA-1= mammalian HIFα/ARNT) predicts.

CKY-1 is a new bHLH-PAS protein that is able to dimerize with AHA-1. Blast searches identified 3 mammalian homologs, including a predicted human open reading frame (ORF) and two murine ESTs. The 5' sequences of the two murine ESTs are different, suggesting that there are at least two mouse CKY-1 homologs. Their bHLH domains are highly homologous (Chapter 3, Figure 6A). Thus, CKY-1 may represent a new bHLH-PAS protein subfamily that is able to form heterodimers with ARNT.

C. elegans aha-1 has an essential role during larval development

Mutants that lack *aha-1* function are larval lethal, and the phenotype is completely penetrant. Phenotypic analysis of the *aha-1* mutants suggested that they are defective in pharynx function. The arrested larvae have pale intestines, similar to the starved animals. The terminally arrested mutant animals have a "stuffed" pharynx phenotype (Chapter 3, Figure 2). The ink particle-feeding assay confirmed that *aha-1* mutants feed poorly. After feeding ink particles for 20 minutes, only about 40% of the mutant animals contain ink in their intestines, while for wild type N2, over 97% of them do (Chapter 3, Figure 2). A *cky-1:aha-1* fusion, pHJ32, in which *cky-1* promoter was placed upstream of the *aha-1* coding sequence,

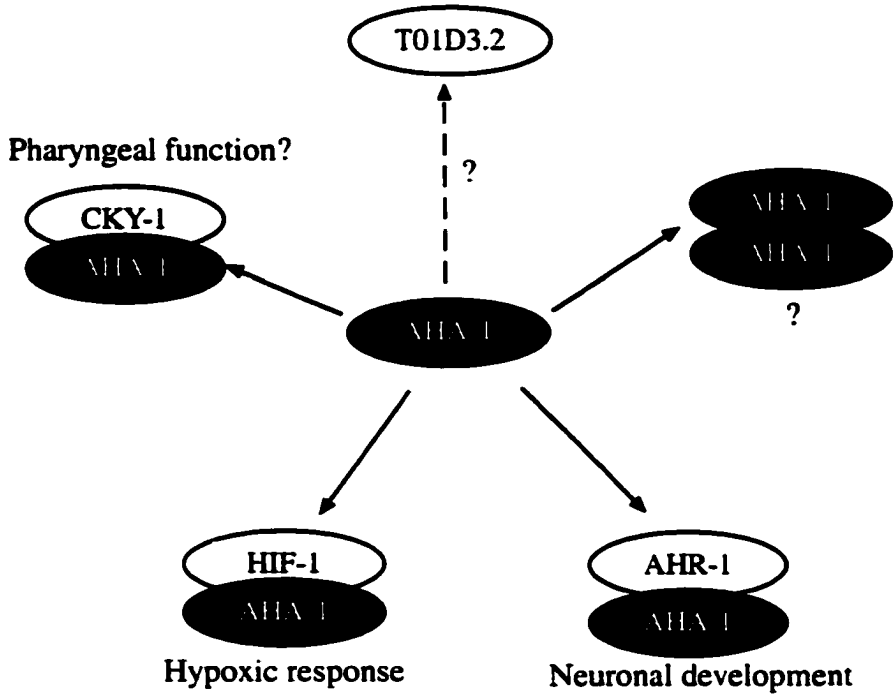

Figure 1 Model for the functions of *C. elegans* bHLH-PAS proteins. *C. elegans* genome encodes 5 bHLH-PAS genes. *aha-1* is the *C. elegans* homolog of mammalian ARNT. AHA-1 is able to dimerize with multiple bHLH-PAS proteins. Its dimerization partners include: mammalian AHR homolog AHR-1, which mediates neuronal development (Powell-Coffman 1998 and Qin and Powell-Coffman, manuscript in preparation), mammalian HIFα homolog HIF-1, which mediates the hypoxic response (Chapter 2) and a new bHLH-PAS protein CKY-1, which has a possible role in pharyngeal function (Chapter 3). AHA-1 also forms homodimers *in vitro*. The function of the homodimers is unclear (Chapter 3). Whether AHA-1 interacts with T01D3.2 is also unclear.

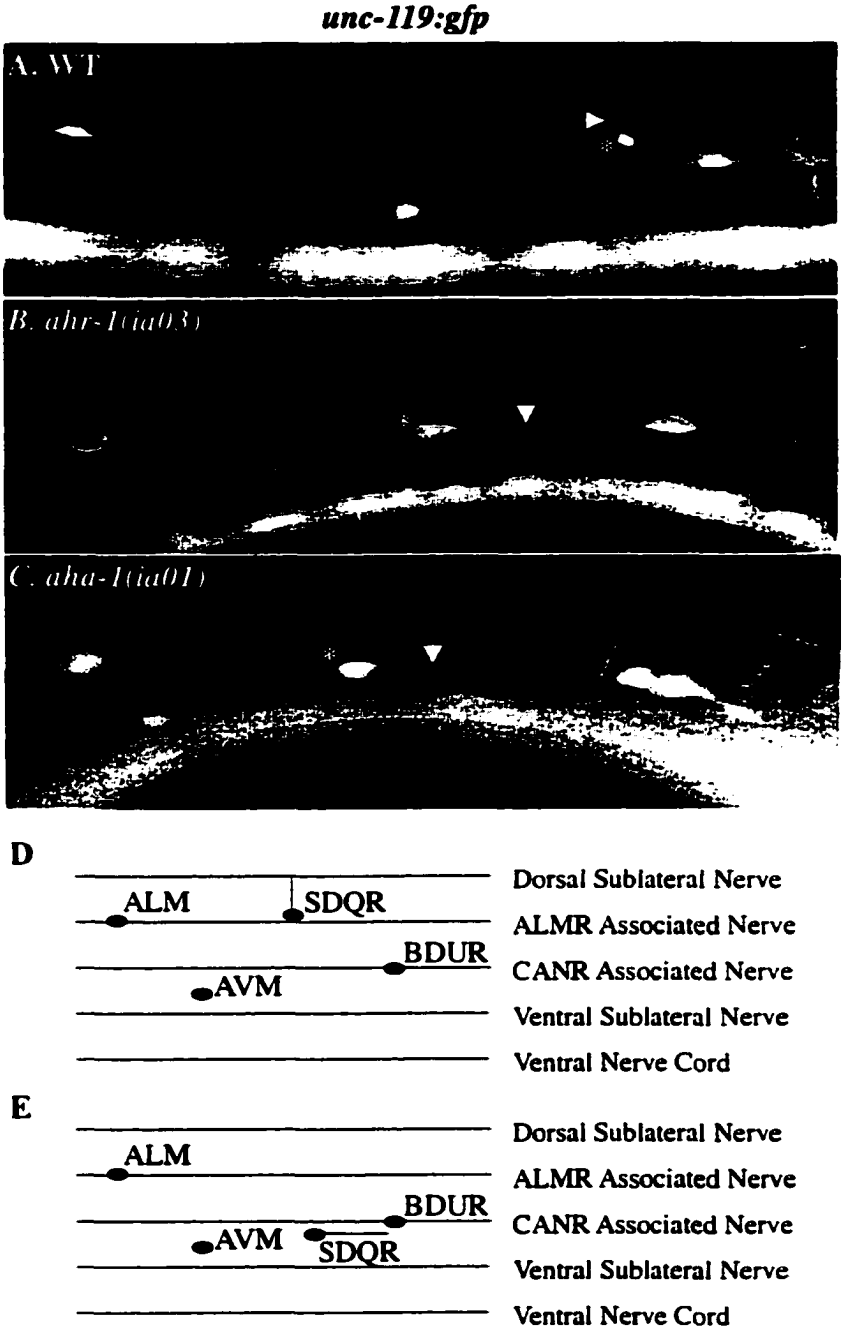

Figure 2 SDQR migration defects in *ahr-1* and *aha-1*-defective animals. A-C. Photographs of neuronal cell bodies and axons along the right anterior body wall. A pan-neuronal marker *unc-119:gfp* were used to reveal the position of the cell bodies and axons. SDQR cell body and axon are indicated by asterisks and arrowheads respectively. A. The position of SDQR cell body and axon in wild-type animals. SDQR axon migrates dorsally from the cell body at the ALMR associated nerve to the dorsal sublateral nerve where it turns anteriorly. B and C. SDQR migration in *ahr-1*-defective (B) and *aha-1*-defective (C) animals. SDQR is positioned ventrally and its axons migrate laterally to the anterior in both *ahr-1* and *aha-1* mutants. D and E. Illustration of A and B/C respectively.

efficiently rescued the *aha-1* mutants to viability. 10H8 immunostaining of the rescued animals demonstrated that AHA-1 is mainly detected in the pharynx and a pair of phasmid neurons in the tail (Chapter 3, Figure 4).

The larval lethal phenotype of *aha-1* mutants can be explained by two non-exclusive models: *aha-1* interacts with *cky-1* and the heterodimer mediates an essential pharyngeal function. Alternatively *aha-1* has an essential function as a homodimer in *cky-1*-expressing cells. I prefer the heterodimer model for the following reasons. 1. AHA-1 forms a heterodimer with CKY-1 *in vitro*, and AHA-1 is nuclear localized in the subset of pharyngeal cells that express the CKY-1:GFP reporter pR15. 2. Expressing *aha-1* from the *cky-1* promoter rescued the *aha-1* larval arrest phenotype. 3. The "stuffed" pharynx phenotype of *aha-1* mutants is very similar to that of *pha-4* temperature-sensitive mutants (7). PHA-4 controls both the early and later stages of pharynx development and is required for the expression of many pharyngeal genes even after the pharynx has formed (7). Multiple PHA-4 binding sites exist in the *cky-1* promoter. PHA-4 may directly control *cky-1* expression to regulate AHA-1/CKY-1 heterodimer activity in the pharynx. If the heterodimer model is correct, then we expect the *cky-1* loss-of-function will result in larval arrest. Wild type animals treated with *cky-1* dsRNA did not reveal any embryonic or postembryonic defects. However this data may not be informative due to the inherent problems with the RNAi technique (8). Resolution of the problem will require genetic analysis of *cky-1* function, achieved by generating a mutation in this gene using reverse genetic methods.

It is worth noting that the *cky-1:aha-1* fusion is also expressed in a pair of phasmid neurons and a couple of cells immediately outside of the pharynx. It is formally possible that the expression of *aha-1* in these cells rescued the mutant phenotype. This could be tested using known promoters to direct pharyngeal specific expression of *aha-1* (such as the pharyngeal muscle specific *myo-2* promoter (9)). We do not yet understand the pharyngeal functions of *aha-1* at the cellular level. However, promoters that direct expression in subsets of pharyngeal cells could be used to refine the cellular requirement of *aha-1* in the pharynx. The promoters of the pharyngeal genes described by Gaudet et al. (2002) could provide valuable reagents for this experiment (7).

The regulation of *cky-1* expression in the pharynx, especially the possibility that PHA-4 may directly regulate *cky-1* expression can be analyzed by promoter truncation, combined with site-specific mutagenesis to mutate the potential PHA-4 binding sites on the *cky-1* promoter. *pha-4* temperature-sensitive mutants can be used to test whether PHA-4 activity is required for the expression of *cky-1:gfp* reporter.

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